AD_____

Award Number: DAMD17-94-J-4185

TITLE: Role of the erbB3 Gene Product in Breast Cancer Cell
Proliferation

PRINCIPAL INVESTIGATOR: John G. Koland, Ph.D.

CONTRACTING ORGANIZATION: University of Iowa
Iowa City, Iowa 52242-1320

REPORT DATE: September 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are
those of the author(s) and should not be construed as an official
Department of the Army position, policy or decision unless so
designated by other documentation.

DTIC QUALITY INSPECTED 4

20001013 112

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 1999	3. REPORT TYPE AND DATES COVERED Final (1-Sep-94 - 31-Aug-99)

4. TITLE AND SUBTITLE
Role of the erbB3 Gene Product in Breast Cancer Cell Proliferation

5. FUNDING NUMBERS
DAMD17-94-J-4185

6. AUTHOR(S)
John G. Koland, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Iowa

Iowa City, Iowa 52242-1320

E-MAIL:
john-koland@uiowa.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The role of the heregulin receptor ErbB3 in breast cancer cell proliferation was examined. In the first year of funding, we discovered that ErbB3, although possessing a protein tyrosine kinase (PTK) homology domain, is in fact devoid of intrinsic PTK activity. In year two, we demonstrated the critical dependence of heregulin signaling upon the PTK activity of ErbB2 and characterized the interaction of ErbB3 with phosphoinositide (PI) 3-kinase in breast cancer cells. In year three, we identified a single tyrosine residue in ErbB3 responsible for binding the Shc adapter protein, clarified the role of Shc in activation of the Ras/mitogen-activated protein kinase (MAPK) pathway, and began an examination of other ErbB3 tyrosine residues. In the fourth year, we implicated six specific ErbB3 tyrosine residues in mediating interactions with PI 3-kinase and activating its downstream signaling target, the protein kinase Akt. In the fifth year of the project, we investigated a novel mechanism by which the Akt kinase was activated in the absence of a direct interaction of PI 3-kinase with either ErbB2 or ErbB3. Subsequently, we compared the respective contributions of the Ras/MAPK and PI 3-kinase signaling pathways in mediating the cell proliferation and transformation responses resulting from ErbB2/ErbB3 activation.

14. SUBJECT TERMS
Tyrosine Kinase, Signal Transduction, ErbB, EGF receptor, Phosphatidylinositide 3-kinase, Growth Factors, Breast Cancer

15. NUMBER OF PAGES
77

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

___ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

___ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

___ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

<table>
<tr><td>9/29/99</td></tr>
<tr><td>PI - Signature</td><td>Date</td></tr>
</table>

TABLE OF CONTENTS

INTRODUCTION

Breast cancer cells express abnormally high levels of receptor proteins in the ErbB family, which includes the epidermal growth factor (EGF) receptor, ErbB2, ErbB3 and ErbB4 (also designated as HER1-HER4, respectively) (**1, 10, 12, 13, 15**). High levels of EGF receptor and ErbB2 expression in tumor cells have been considered indicators of poor prognosis (**5, 13**). Given that ErbB family receptors, when activated either by their polypeptide ligands or by oncogenic mutations, stimulate cell proliferation and transformation *in vitro*, it appears likely that these receptors when overexpressed might play significant roles in the pathogenesis of human breast cancer. The polypeptide heregulin (also designated as neu differentiation factor and glial cell growth factor) is secreted from breast cancer cells (**4**) and has been shown to activate the ErbB2, ErbB3 and ErbB4 receptor proteins. Hence it is possible that heregulin and ErbB receptor proteins function via an autocrine mechanism to augment the proliferation of breast cancer cells, much as the EGF receptor ligand transforming growth factor α (TGF-α) has been proposed to do in the context of ovarian cancer (**11**). Recently, ErbB2 and ErbB3 have been shown to function as a coreceptor for heregulin (**16**). Elucidation of the signaling mechanisms of this unique ErbB coreceptor complex has become a major goal of our laboratory.

In the first year of funding, we demonstrated convincingly that the ErbB3 protein, in contrast to the other ErbB family members, is actually devoid of intrinsic protein tyrosine kinase (PTK) activity (**14**). This and subsequent studies were facilitated by our cloning of the rat ErbB3 cDNA in this period (**3**). In the second year of funding, we showed that the PTK activity necessary for the phosphorylation of the two constituents of the ErbB2/ErbB3 coreceptor complex and the subsequent activation of downstream signaling events is actually provided the ErbB2 PTK (**8**). Also, the yeast two-hybrid system was used to investigate the mechanism of interaction between the ErbB3 protein and the signal-transducing enzyme phosphoinositide (PI) 3-kinase (**2**). Efforts in the third year of funding focussed on examining the mechanism of activation of the Ras/mitogen-activated protein kinase (MAPK) pathway by the ErbB2/ErbB3 heregulin coreceptor (**17**). In the fourth year of funding, we successfully completed and published studies initiated in the second and third years of funding (**2, 8, 17**). We also initiated our investigations of the downstream signaling events activated by ErbB2/ErbB3 coreceptor-associated PI 3-kinase, studies that were also pursued in the fifth year of the project. Investigations performed in the fifth year of the project, which was the year of

an awarded no-cost extension of the original four-year project, are detailed below. The main goals in this final year of the project were to conclude our studies of the downstream signaling events activated by PI 3-kinase and to examine the respective roles of the Shc/MAPK and PI 3-kinase signaling pathways in promoting the proliferation and transformation of cells expressing ErbB2/ErbB3 coreceptors. These studies have shed significant light upon the mechanisms by which ErbB coreceptors might function to augment the cancerous transformation of mammary tissue.

BODY

Our previous progress report described our initial investigations of the PI 3-kinase signaling pathway and its mechanism of activation by the ErbB2/ErbB3 heregulin coreceptor. We continued to pursue this problem in the final year of the project. A key unanswered question resulting from our preliminary work concerned the mechanism of activation of the Ser/Thr kinase Akt, presumed to be a key signaling target of activated PI 3-kinase. We had documented the heregulin- and ErbB2/ErbB3-dependent activation of Akt. However, when investigating the signaling functions of ErbB3-6F, a mutant ErbB3 receptor that failed to bind PI 3-kinase, we still observed a heregulin-dependent Akt activation. We ruled out the possibility that the ErbB2/ErbB3-6F coreceptor recruited PI 3-kinase via its binding to the ErbB2 protein by selectively immunoprecipitating this coreceptor constituent. Hence, the question remained whether PI 3-kinase and its target Akt were activated by a novel mechanism not involving the direct association of PI 3-kinase with the ErbB2/ErbB3 coreceptor.

In the final funding period, we first examined the involvement of PI 3-kinase in the heregulin- and ErbB2/ErbB3 coreceptor-dependent activation of Akt by use of the PI 3-kinase inhibitor wortmannin. Wortmannin clearly abolished Akt activation (see Figure 1), which strongly suggested that PI 3-kinase was involved in the activation of Akt. However, the PI 3-kinase inhibitor wortmannin is not entirely specific and could conceivably have inhibited another enzyme that mediated Akt activation in this system. It was therefore desirable to compare the abilities of the ErbB2/ErbB3 and ErbB2/ErbB3-6F coreceptors to activate PI 3-kinase. An indirect means of assessing PI 3-kinase activation in cells is to perform PI 3-kinase activity assays on antiphosphotyrosine immunoprecipitates (6). The logic here is that a number of protein substrates are known to interact with and activate PI 3-kinase when they are phosphorylated on tyrosine residues. In these experiments, we detected PI 3-kinase activity in antiphosphotyrosine immunoprecipitates from cells expressing the

ErbB2/ErbB3 coreceptor, which was consistent with the observed association of PI 3-kinase with the phosphorylated ErbB3 protein (6). However, no PI 3-kinase activity was seen in immunoprecipitates from cells expressing the ErbB2/ErbB3-6F coreceptor. This suggested that PI 3-kinase was activated by a mechanism not involving its association with a substrate phosphorylated on tyrosine residues. One possibility was that the Ras protein, which is activated by the ErbB2/ErbB3-6F coreceptor complex, directly interacted with and activated PI 3-kinase, a mechanism that would not require association of PI 3-kinase with the coreceptor or another phosphorylated substrate. To this end, we immunoprecipitated the Ras protein and assayed these immunoprecipitates for associated PI 3-kinase activity. No PI 3-kinase activity was detected in Ras immunoprecipitates from heregulin-stimulated cells expressing the ErbB2/ErbB3 coreceptor, which indicated that, if PI 3-kinase was indeed activated by the ErbB2/ErbB3-6F coreceptor, it occurred via a novel mechanism not involving the association of PI 3-kinase with either Ras or a phosphorylated protein substrate.

A key experiment necessary to clarify this issue was to directly assay PI 3-kinase activity in intact cells expressing the wild-type and mutant ErbB coreceptors. At the end of the funding period, we developed such an assay, which relied on the use of fluorescent reporter molecule that binds the PI 3-kinase product phosphatidylinositol-(3,4,5)-trisphosphate (PIP_3) produced in cells. A PIP_3 reporter previously described by Kontos and colleagues (9) consists of the PIP_3-binding pleckstrin homology (PH) domain of the Akt kinase fused to the green fluorescent protein (GFP). A cDNA encoding this fusion protein (kindly provided by C. D. Kontos and K. G. Peters) was subcloned into the pcDNA3 expression vector, and the resulting construct (pcDNA3-GFP-PH) was transfected into COS7 and NIH-3T3 cells expressing ErbB2 and ErbB3 receptor proteins. The subcellular localization of the expressed GFP-PH domain was examined by use of confocal fluorescence microscopy (see Figure 2). It can be seen that while there was a diffuse cytoplasmic localization of GFP-PH in the absence of receptor stimulation (Figure 2A), stimulation of the cells with heregulin resulted in a redistribution of the fluorescent reporter to the cytoplasmic membrane of the cells (Figure 2B). Kontos and colleagues have shown that this redistribution of the reporter is correlated with the production of PIP_3 in the cytoplasmic membrane in response to PI 3-kinase activation (9). We are currently using this assay of PI 3-kinase activation in intact cells to compare the abilities of the ErbB2/ErbB3 and ErbB2/ErbB3-6F coreceptors to activate this signaling enzyme. A manuscript describing the most significant of our findings regarding the activation of the PI 3-kinase signaling system by

the ErbB2/ErbB3 coreceptor is in preparation (N. J. Hellyer and J. G. Koland, manuscript in preparation).

Another avenue of research taken in the final year of funding was a comparison of the respective roles of the Ras/MAPK and PI 3-kinase signaling pathways in the stimulation of cellular proliferation and transformation that is mediated by the ErbB2/ErbB3 heregulin coreceptor. The results of these studies will be highly relevant in the context of human breast cancer, as it has been proposed that overexpression of these receptor proteins is involved in the cancerous transformation of mammary tissue. We had in previous funding periods generated two ErbB3 mutants, ErbB3-Y1325F and ErbB3-6F, which individually fail to activate either the Ras/MAPK or PI 3-kinase signaling pathways, respectively [see (17) and Annual Progress Report, August 1998]. We now generated a third mutant receptor, ErbB3-7F, that activated neither pathway. Our goal was to compare the abilities of ErbB2/ErbB3 coreceptors incorporating either wild-type ErbB3 (ErbB3-WT), ErbB3-Y1325F, ErbB3-6F or ErbB3-7F to mediate the stimulation of cellular DNA synthesis and cellular transformation. Cellular DNA synthesis was measured by [^{3}H]-thymidine incorporation assays, the representative results of which are shown in Figure 3. It was observed that ErbB2/ErbB3 coreceptors incorporating ErbB3-WT mediated a strong heregulin-dependent increase in DNA synthesis. Coreceptors incorporating the ErbB3-Y1325F mutant, which fails to activate the Ras/MAPK signaling pathway, showed a modestly attenuated response. In contrast, coreceptors incorporating the ErbB3-6F mutant, which does not directly interact with the PI 3-kinase signaling enzyme, showed a strongly attenuated DNA synthesis response to heregulin. The ErbB3-7F mutant, which directly activates neither the Ras/MAPK nor the PI 3-kinase signaling pathway, showed no capacity to mediate a heregulin-dependent DNA synthesis response.

The failure of the ErbB3-7F mutant to mediate any mitogenic response suggests that activation of either the Ras/MAPK or PI 3-kinase signaling pathway is necessary for this response, although it is possible that the seven Tyr→Phe substitutions occurring in this receptor mutant also attenuate the activation of some as yet unidentified mitogenic signaling pathway. Assuming this not to be the case, we can also conclude that (1) both the Ras/MAPK and PI 3-kinase pathways contribute to the mitogenic capacity of the ErbB2/ErbB3 coreceptor and (2) the PI 3-kinase pathway plays a more dominant role in this response.

We next wished to compare the transforming potentials of the various ErbB3 proteins. In this case, a convenient and often used assay of transformation, the soft-agar colony formation assay, was employed. Representative results of these

experiments are given in Figure 4. It was observed that expression of ErbB3-WT in NIH-3T3 fibroblasts in the context of an ErbB2/ErbB3 coreceptor strikingly enhanced the ability of the fibroblasts to form colonies in soft agar. Interestingly, although this colony formation showed a modest heregulin-dependence, a large number of colonies were formed in the absence of heregulin. This high basal rate of colony formation suggested that either the ErbB2/ErbB3 coreceptor complex is intrinsically active in the absence of ligand-stimulation or the coreceptor in NIH-3T3 fibroblasts is stimulated by the production of endogenous heregulin in these cells. Notably, the colony formation mediated by the ErbB3-Y1325F receptor, which fails to activate the Ras/MAPK pathway, was not at all attenuated relative to the wild-type receptor. In contrast, the colony-forming ability of fibroblasts expressing the ErbB3-6F receptor, which does not interact with the PI 3-kinase signaling enzyme, was markedly reduced.

The results of these colony forming assay suggest that (1) activation of either the Ras/MAPK or PI 3-kinase signaling pathway is necessary for the induction of cellular transformation by the ErbB2/ErbB3 coreceptor, (2) the Ras/MAPK pathway makes a minimal contribution to the transforming potential of the coreceptor, and (3) the PI 3-kinase signaling pathway makes a dominant contribution. These findings are reminiscent of the results of our DNA synthesis (mitogenesis) assays (c.f. Figure 3), in which the PI 3-kinase signaling pathway made a stronger contribution than the Ras/MAPK pathway to the stimulation of DNA synthesis by the ErbB2/ErbB3 coreceptor. However, the dominance of the PI 3-kinase pathway appeared to be more marked in the case of the cellular transformation assay. The results of these comparisons of the Ras/MAPK and PI 3-kinase pathways in coreceptor signaling are the basis of a manuscript that is now in preparation (U. Vijapurkar and J. G. Koland, manuscript in preparation). In the final funding period, we also submitted an invited review article summarizing the recent research findings of our and other laboratories regarding the signaling mechanisms and biologic functions of the ErbB3 protein (U. Vijapurkar, H.-H. Kim, and J. G. Koland, in press).

In the past five years of research, we have been quite successful in pursuing the Specific Aims of the original proposal. As described in our annual progress reports and with the concurrence of the reviewers of these reports, two major strategic changes have been made along the way. First, the goal of Specific Aim 1, which was to identify kinase-activating mutations in the ErbB3 protein, was made largely obsolete by our discovery that the ErbB3 protein, unlike other ErbB family members, in fact possesses no intrinsic kinase activity (**14**). As a consequence, more emphasis was placed upon the biochemical characterization of the ErbB3 protein (**14**) and the mechanism of its

phosphorylation by the ErbB2 and EGF receptor proteins (**7**). Second, with the general acceptance in the field now being that the Ser/Thr kinase Akt is a more important target of activated PI 3-kinase than is protein kinase Cζ, we altered the studies of Specific Aim 5 to make it instead an investigation of the Akt kinase. The pursuit of both of these modified Specific Aims, as did that of the remaining Specific Aims, led to significant findings that were consistent with the overall goal of the proposal: to investigate the potential contribution of the novel ErbB3 protein in breast cancer cell proliferation. One shortcoming of our work, is that it relied heavily upon model cultured cell systems and a limited number of assays of mitogenesis and transformation, which was necessary to obtain a clear delineation of the roles of each ErbB coreceptor constituent and specific signaling molecules. It would be important to extend these studies of breast cancer cell proliferation and transformation by the use of other assays of cell transformation such as cell migration assays and nude-mouse models of tumor cell colonization and growth. This said, we feel that we have made a quite significant contribution to our understanding of the signal transduction mechanisms of the ErbB2/ErbB3 protein, a receptor system that continues to be of great significance in terms of the etiology, diagnosis, and, most recently, the treatment of human breast cancer.

KEY RESEARCH ACCOMPLISHMENTS

- Cloning of the rat ErbB3 cDNA from a rat liver cDNA library
- Expression and purification of a recombinant ErbB3 cytoplasmic domain protein
- Biochemical characterization of the ErbB3 protein
- Demonstration of the lack of intrinsic protein tyrosine kinase activity in ErbB3
- Characterization of EGF receptor- and ErbB2-mediated phosphorylation of ErbB3
- Identification of the Shc binding site on the ErbB3 C-terminus
- Investigation of the mechanism of activation of the Shc/Ras/MAPK signaling pathway by the ErbB2/ErbB3 coreceptor
- Identification of multiple PI 3-kinase binding sites on the ErbB3 C-terminus
- Investigation of the mechanism of activation of the PI 3-kinase/Akt signaling pathway by the ErbB2/ErbB3 coreceptor
- Generation of mutant ErbB3 receptor proteins that selectively activate either the Ras/MAPK or PI 3-kinase signaling pathway
- Evaluation of the respective roles of the Ras/MAPK and PI 3-kinase signaling pathways in the mediation of proliferative and transforming responses to ErbB coreceptor activation

REPORTABLE OUTCOMES

Manuscripts:

1. Vijapurkar, U., Kim H.-H., and Koland, J.G. The ErbB3/HER3 receptor protein: signaling mechanisms and biologic function. Current Topics in Biochemical Research, in press.

2. Hellyer, N.J. and Koland, J.G. Heregulin-dependent activation of PI 3-kinase and Akt via the ErbB2/ErbB3 coreceptor. In preparation.

3. Vijapurkar, U.P., Hellyer, N.J., Kratz, D.A., and Koland, J.G. Role of PI 3-kinase and MAPK pathways in heregulin-stimulated mitogenesis by the ErbB2/ErbB3 coreceptor complex. In preparation.

Abstracts:

1. Vijapurkar, U.P., Hellyer, N.J., Kratz, D.A., and Koland, J.G. Role of PI 3-kinase and MAPK pathways in heregulin-stimulated mitogenesis by the ErbB2/ErbB3 coreceptor complex. Molecular Targets for Cancer, Houston TX, October, 1998.

Presentations:

1. Koland, J.G. Signaling mechanisms of ErbB2/ ErbB3 heregulin coreceptors. Department of Molecular Biology, Vanderbilt University, Nashville, TN, August, 1998.

Patents and licenses:

None.

Degrees obtained:

1. Hong-Hee Kim   Ph.D.

2. Nathan J. Hellyer   Ph.D.

3. Ulka Vijapurkar   Ph.D.

4. Morven Shearer   M.S.

Funding applied for:

1. DOD Breast Cancer Research Program IDEA Award
    PI: J. G. Koland
    Title: Inhibition of breast cancer proliferation with dominant-negative receptors and heregulin antisense DNAs
    Applied: 1997. Not funded.

2. DOD Breast Cancer Research Program Predoctoral Traineeship
    PI: M. C. Shearer   Mentor: J. G. Koland
    Title: Regulatory pathways involved in heregulin-induced proliferation and differentiation of human breast cancer cells
    Funded: 4/6/98-5/5/01

Employment:

1. Hong-Hee Kim, Ph.D.   Assistant Professor
    Department of Immunology, Chosun University Dental College, Kwanju, Korea

2. Ulka Vijapurkar, Ph.D.   Postdoctoral Fellow
    Department of Dermatology, University of California School of Medicine, San Francisco, CA

CONCLUSIONS

The research we have performed in the past five years was spawned by the previous discovery that ErbB3, a novel member of the ErbB receptor family, is expressed at high levels in a subset of human breast cancer specimens and appears to be activated in these tumor cells. The roles in breast cancer pathogenesis of two other ErbB family members, the EGF receptor and ErbB2, had already been extensively investigated, and indeed the ErbB2 protein was being examined as a target for a novel therapeutic agent, Herceptin. (Herceptin, a monoclonal antibody recognizing the ErbB2 protein, has recently been approved for use in treatment of metastatic breast cancer.) The observed phosphorylation and activation of the ErbB3 protein in some breast cancer cells suggested that ErbB3 might be function to augment the growth and metastasis of these cells. Through the course of our subsequent efforts, we have answered important questions regarding the mechanism of activation of the ErbB3 protein, the nature of its intracellular signaling targets, and the abilities of distinct ErbB3-activated signaling pathways to stimulate cell division (mitogenesis) and transformation. Key results of this work include: (1) the discovery that the ErbB3 protein is devoid of intrinsic kinase activity and must work in cooperation with another ErbB family coreceptor partner such as ErbB2; (2) the identification of the Shc/MAPK and PI 3-kinase signaling pathways as the major mediators of mitogenic signals from the ErbB2/ErbB3 coreceptor; (3) the identification of the PI 3-kinase pathway as the dominant pathway eliciting a cell transformation response to ErbB2/ErbB3 activation. As the ErbB2 protein has recently been shown to be an effective target for a novel breast cancer therapeutic agent, understanding the interaction and cooperation of the ErbB2 and ErbB3 proteins in the context of cell growth control is an important goal, which could lead to the improvement and further development of such therapeutic agents. Our characterization of the signaling pathways activated by ErbB2/ErbB3 and of their roles in the stimulation of cell proliferation and transformation could ultimately improve our understanding of how such therapeutic agents can stop or even reverse tumor cell growth and could possibly lead to the identification of new intracellular targets for therapeutic agents.

REFERENCES

1. Gullick, W. J. 1990. 4. The role of the epidermal growth factor receptor and the c-erbB-2 protein in breast cancer. Int. J. Cancer **46:**55-61.

2. Hellyer, N. J., K. Cheng, and J. G. Koland. 1998. ErbB3 (HER3) interaction with the p85 regulatory subunit of phosphoinositide 3-kinase. Biochem. J. **333:**757-763.

3. Hellyer, N. J., H.-H. Kim, C. H. Greaves, S. L. Sierke, and J. G. Koland. 1995. Cloning of the rat ErbB3 cDNA and characterization of the recombinant protein. Gene **165:**279-284.

4. Holmes, W. E., M. X. Sliwkowski, R. W. Akita, W. J. Henzel, J. Lee, J. W. Park, D. Yansura, N. Abadi, H. Raab, G. D. Lewis, H. M. Shepard, W. J. Kuang, W. I. Wood, D. V. Goeddel, and R. L. Vandlen. 1992. Identification of heregulin, a specific activator of p185^{erbB2}. Science **256:**1205-1210.

5. Jain, S., M. I. Filipe, W. J. Gullick, J. Linehan, and R. W. Morris. 1991. c-erbB-2 proto-oncogene expression and its relationship to survival in gastric carcinoma: An immunohistochemical study on archival material. Int. J. Cancer **48:**668-671.

6. Kim, H.-H., S. L. Sierke, and J. G. Koland. 1994. Epidermal growth factor-dependent association of phosphatidylinositol 3-kinase with the erbB3 gene product. J. Biol. Chem. **269:**24747-24755.

7. Kim, H.-H., U. Vijapurkar, N. J. Hellyer, D. Bravo, and J. G. Koland. 1997. Signal transduction by epidermal growth factor and heregulin via the kinase-deficient ErbB3 protein. Biochem. J. **334:**189-195.

8. Kim, H.-H., U. Vijapurkar, N. J. Hellyer, D. Bravo, and J. G. Koland. 1998. Signal transduction by epidermal growth factor and heregulin via the kinase-deficient ErbB3 protein. Biochem. J. **334:**189-95.

9. Kontos, C. D., T. P. Stauffer, W. P. Yang, J. D. York, L. Huang, M. A. Blanar, T. Meyer, and K. G. Peters. 1998. Tyrosine 1101 of Tie2 is the major site of association of p85 and is required for activation of phosphatidylinositol 3-kinase and Akt. Mol. Cell. Biol. **18:**4131-40.

10. Kraus, M. H., W. Issing, T. Miki, N. C. Popescu, and S. A. Aaronson. 1989. Isolation and characterization of *ERBB3*, a third member of the *ERBB*/epidermal

growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. Proc. Natl. Acad. Sci. USA **86:**9193-9197.

11. Kurachi, H., K. Morishige, K. Amemiya, H. Adachi, K. Hirota, A. Miyake, and O. Tanizawa. 1991. Importance of transforming growth factor alpha/epidermal growth factor receptor autocrine growth mechanism in an ovarian cancer cell line in vivo. Cancer Res. **51:**5956-5959.

12. Lovekin, C., I. O. Ellis, A. Locker, J. Robertson, J. Bell, R. Nicholson, W. J. Gullick, C. W. Elston, and R. W. Blamey. 1991. c-erbB-2 oncoprotein expression in primary and advanced breast cancer. Br. J. Cancer **63:**439-443.

13. Sainsbury, J. R. C., J. R. Fardon, G. K. Needham, A. J. Malcom, and A. L. Harris. 1987. Epidermal growth factor receptor status as a predictor of early recurrence of and death from breast cancer. Lancet **1:**1398-1402.

14. Sierke, S. L., K. Cheng, H.-H. Kim, and J. G. Koland. 1997. Biochemical characterization of the protein tyrosine kinase homology domain of the ErbB3 (HER3) receptor protein. Biochem. J. **322:**757-763.

15. Slamon, D. J., W. Godolphin, L. A. Jones, J. A. Holt, S. G. Wong, D. E. Keith, W. J. Levin, S. G. Stuart, J. Udove, A. Ullrich, and M. F. Press. 1989. Studies of the HER-2/*neu* Proto-oncogene in Human Breast and Ovarian Cancer. Science **244:**707-712.

16. Sliwkowski, M. X., G. Schaefer, R. W. Akita, J. A. Lofgren, V. D. Fitzpatrick, A. Nuijens, B. M. Fendly, R. A. Cerione, R. L. Vandlen, and K. L. Carraway, III. 1994. Coexpression of *erb*B2 and *erb*B3 proteins reconstitutes a high affinity receptor for heregulin. J. Biol. Chem. **269:**14661-14665.

17. Vijapurkar, U., K. Cheng, and J. G. Koland. 1998. Mutation of a Shc binding site tyrosine residue in ErbB3/HER3 blocks heregulin-dependent activation of mitogen-activated protein kinase. J. Biol. Chem. **273:**20996-1002.

APPENDICES

Wortmannin: —   100 nM 500 nM
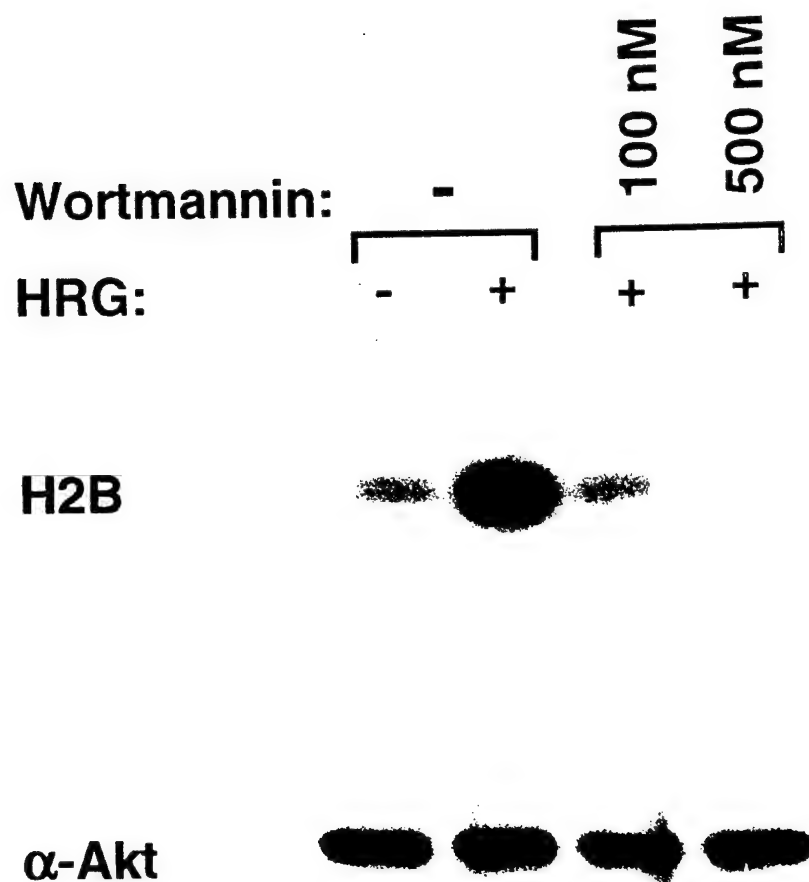
HRG: —   +   +   +

H2B

α-Akt

Figure 1. **Heregulin-dependent Akt activation in the presence of the PI 3-kinase inhibitor wortmannin.** COS7 cells were transfected with ErbB2, ErbB3, and HA-tagged Akt expression plasmids. Cells were preincubated for 30 min with DMSO vehicle or wortmannin (100 or 500 nM), and subsequently treated for 30 min in the presence (+) or absence (-) of 1 nM heregulin. Cells were lysed and lysates immunoprecipited with a hemagglutinin (HA) antibody. Immunoprecipitates were analyzed for Akt activity by *in vitro* protein kinase assays employing histone 2B (H2B) as a substrate, and immunoprecipitated HA-Akt was detected with an Akt-specific antibody (α-Akt). These results are representative of three independent experiments.
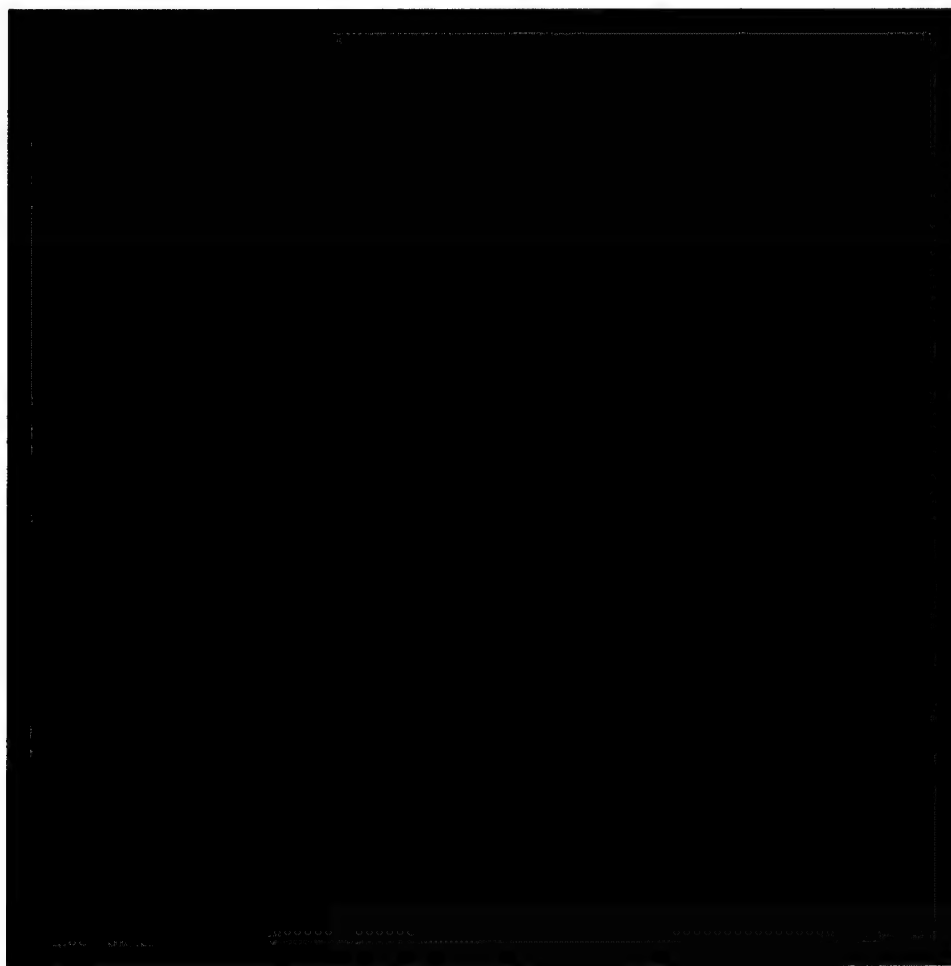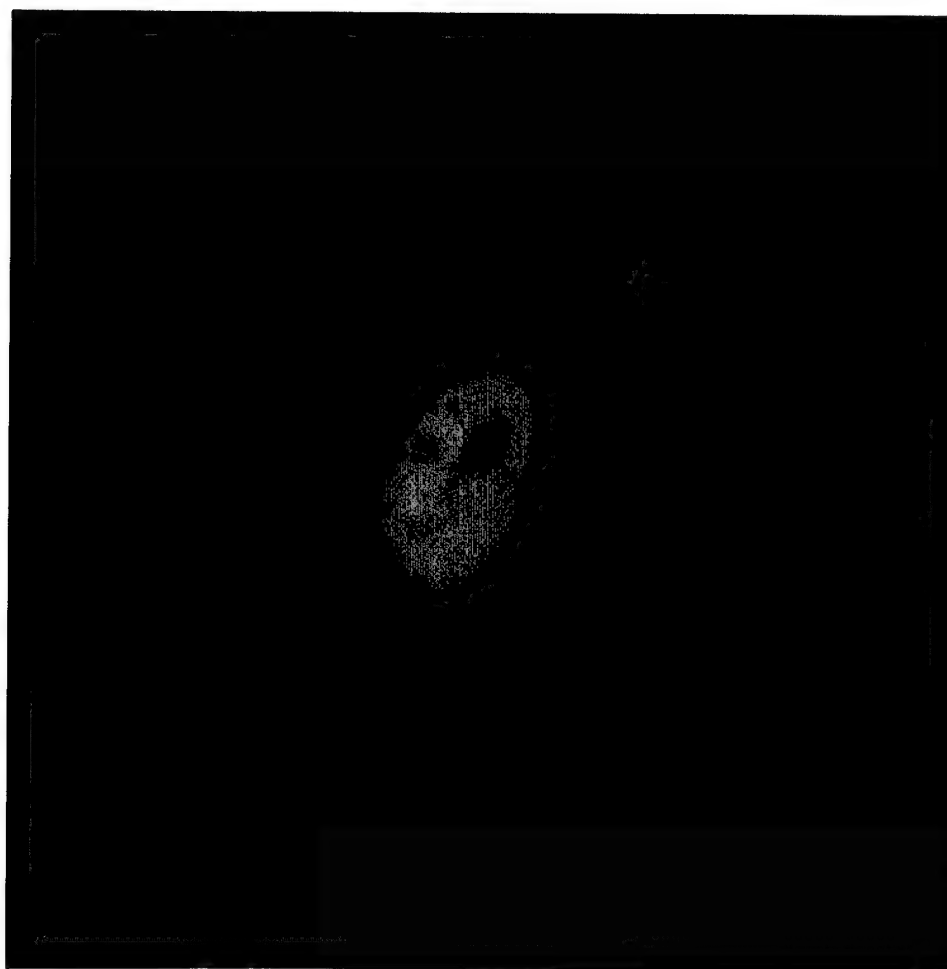
Figure 2A. **PI 3-kinase activation by the ErbB2/ErbB3 heregulin coreceptor assayed in intact cells.** NIH-3T3 cells were transfected with pcDNA3-based vectors incorporating the cDNAs for ErbB2, ErbB3 and the GFP-PH reporter. Twenty-four hours post-transfection, the subcellular localization of the GFP-PH reporter was observed by laser scanning confocal microscopy. Shown in this figure are representative cells not stimulated with heregulin. Note the diffuse cytoplasmic localization of the GFP-PH reporter and minimal fluorescence on the cell periphery (cytoplasmic membrane). Some artifactual nuclear staining is also evident.

Figure 2B. **PI 3-kinase activation by the ErbB2/ErbB3 heregulin coreceptor assayed in intact cells.** NIH-3T3 cells treated as described in Figure 2A were stimulated for 30 min with 1 nM heregulin prior to imaging by laser scanning confocal microscopy. In the representative cell shown here, the GFP-PH reporter localized in the cell periphery, which was indicative of the production of the PI 3-kinase product phosphatidylinositol-(3,4,5)-trisphosphate in the cytoplasmic membrane.
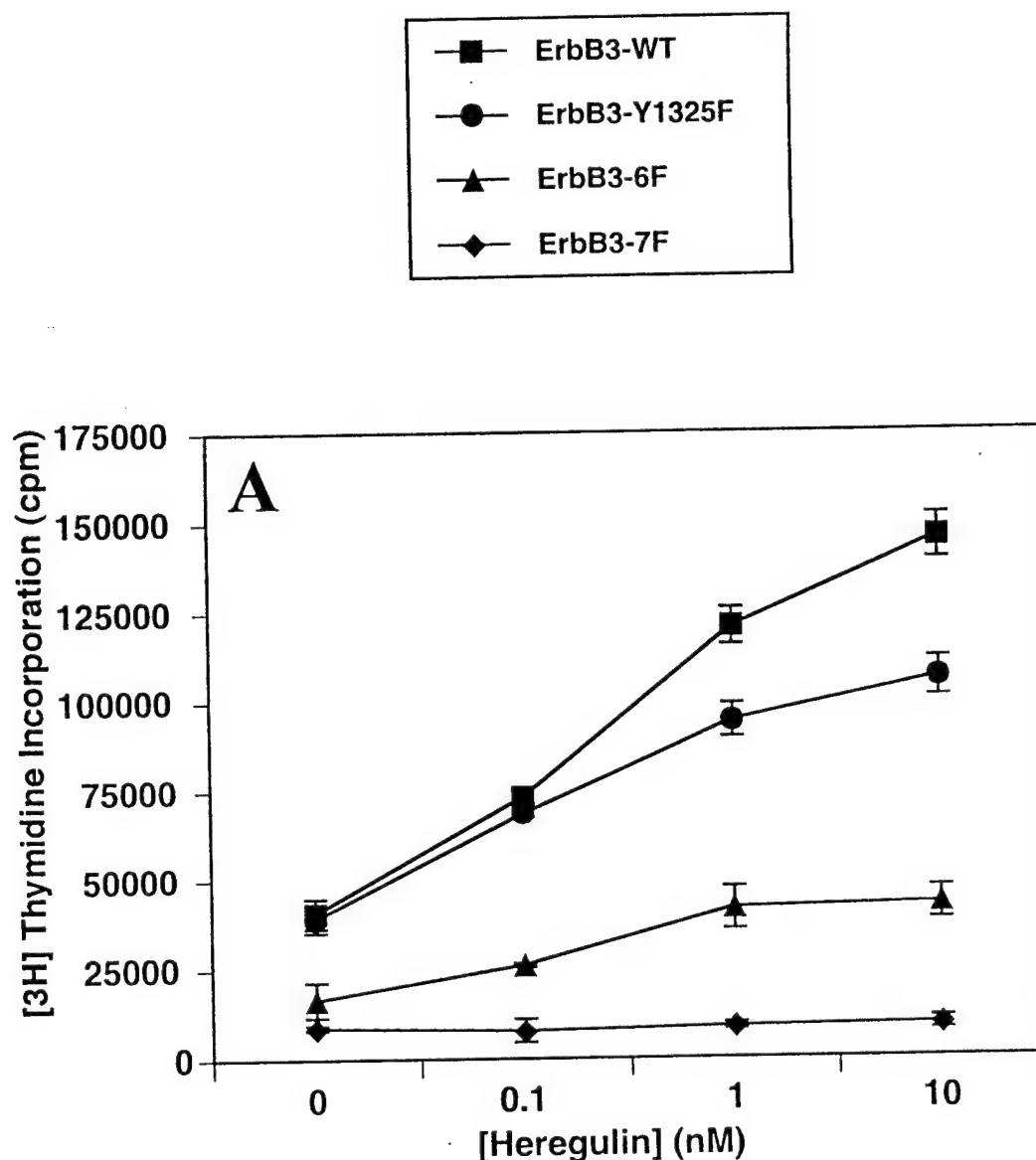
Figure 3. Heregulin-stimulated [³H]thymidine uptake by NIH-3T3 cell lines stably expressing ErbB2/ErbB3 coreceptors. Clonal cell lines expressing high levels of either the wild-type ErbB3 (ErbB3-WT), ErbB3-Y1325F, ErbB3-6F or ErbB3-7F along with endogenous ErbB2 were serum-starved overnight followed by treatment with varying concentrations of heregulin (0-10 nM) for 18 h. [Methyl-³H]thymidine was then added to the stimulation medium, and its incorporation into DNA determined after 4 h. Error bars represent the standard deviation of triplicate assays.
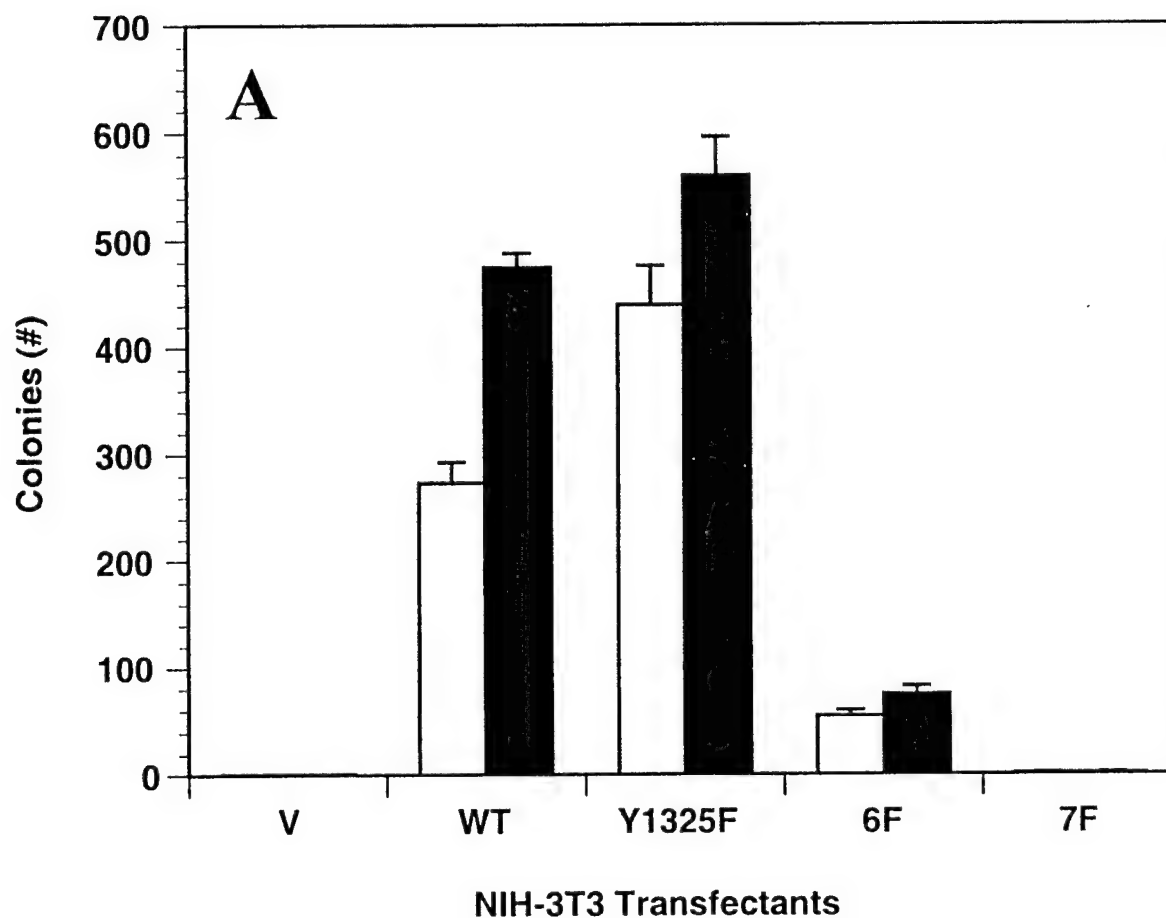
Figure 4. Heregulin-induced colony formation in soft agar by NIH-3T3 cell lines stably expressing ErbB3 receptors. NIH-3T3 cell lines expressing high levels of either the wild-type or a mutant ErbB3 receptor along with endogenous ErbB2 were suspended in 0.35% agar in growth medium supplemented with 10% fetal calf serum and plated (1500 cells per 35 mm dish) over a base layer of 0.5% agar in growth medium supplemented with 10% fetal calf serum and either 1 nM heregulin (filled bars) or control vehicle (open bars). Error bars represent the standard deviation of triplicate assays.

BIBLIOGRAPHY FOR THE FINAL PROGRESS REPORT

Manuscripts:

1. Kim, H.-H., Sierke, S.L., and Koland, J.G. Epidermal growth factor-dependent association of phosphatidylinositol 3-kinase with the ErbB3 gene product. J. Biol. Chem. **269**: 24747-24755, 1994.

2. Gamett, D.C., Greene T., Wagreich, A.R., Kim, H.-H., Koland, J.G. and Cerione R.A. Heregulin-stimulated signaling in rat pheochromocytoma cells: Evidence for ErbB3 interactions with Neu/ErbB2 and p85. J. Biol. Chem. **270**: 19022-19027, 1995.

3. Hellyer, N.J., Kim, H.-H., Greaves, C.H., Sierke, S.L. and Koland, J.G. Cloning of the rat ErbB3 cDNA and characterization of the recombinant protein. Gene **165**: 279-284, 1995.

4. Sierke, S.L., Cheng, K., Kim, H.-H., and Koland, J.G. Biochemical characterization of the protein tyrosine kinase homology domain of the ErbB3 (HER3) receptor protein. Biochem. J. **322**: 757-763, 1997.

5. Hellyer, N.J., Cheng, K., and Koland, J.G. ErbB3 (HER3) interaction with the p85 regulatory subunit of phosphoinositide 3-kinase. Biochem. J. **333**: 757-763, 1998.

6. Kim, H.-H., Vijapurkar, U., Hellyer, N.J., Bravo, D., and Koland, J.G. Signal transduction by epidermal growth factor and heregulin via the kinase-deficient ErbB3 protein. Biochem. J. **334**: 189-195, 1998.

7. Vijapurkar, U., Cheng, K., and Koland, J.G. Mutation of a Shc binding site tyrosine residue in ErbB3/HER3 blocks heregulin-dependent activation of mitogen-activated protein kinase. J. Biol. Chem. **273**: 20996-21002, 1998.

8. Vijapurkar, U., Kim H.-H., and Koland, J.G. The ErbB3/HER3 receptor protein: signaling mechanisms and biologic function. Current Topics in Biochemical Research, in press.

Abstracts:

1.   Kim, H.-H., Sierke, S.L., and Koland, J.G. Signal transduction by the erbB3 gene product: a putative receptor protein tyrosine kinase. Experimental Biology 1994. Anaheim, CA, April, 1994.

2.   Koland, J.G., Kim, H.-H., and Sierke, S.L. Phosphorylation of the erbB3/HER3 gene product by the epidermal growth factor receptor. ASBMB. Washington, D.C., May, 1994.

3.   Kim, H.-H., Hellyer, N.J., Sierke, S.L., and Koland, J.G. Epidermal growth factor-stimulated phosphorylation of the kinase-deficient ErbB3/HER3 protein. ASBMB, San Francisco, CA, May 1995.

4.   Hellyer, N.J., Kim, H.-H., Greaves, C.H., Sierke, S.L., and Koland, J.G. Molecular cloning of the rat *erbB*3 cDNA and characterization of the gene product. ASBMB, San Francisco, CA, May 1995.

5.   Shearer, M.C., Kim, H.-H., Kratz, D.A., and Koland, J.G. Heregulin-dependent activation of MAP kinases in cultured rat astrocytes. ASBMB, New Orleans, LA, June 1996.

6.   Hellyer, N.J. and Koland, J.G. Characterizing the interaction between ErbB3/HER3 and the p85 subunit of phosphatidylinositol 3-kinase with yeast two-hybrid system. ASBMB, New Orleans, LA, June 1996.

7.   Vijapurkar, U.P. and Koland, J.G. Mutation of a Shc binding site tyrosine residue in ErbB3/HER3 blocks heregulin-dependent activation of mitogen-activated protein kinase. ASBMB, San Francisco, CA, August, 1997.

8.   Kratz, D. and Koland, J.G. Growth inhibition of fibroblasts expressing the ErbB3/HER3 receptor protein by heregulin-neutralizing antibody. Growth Factor Signal Transduction Conference, Iowa State University, Ames, IA, September, 1997.

9.   Vijapurkar, U.P. and Koland, J.G. Mutation of a Shc binding site tyrosine residue in ErbB3/HER3 blocks heregulin-dependent activation of mitogen-activated protein kinase. Growth Factor Signal Transduction Conference, Iowa State University, Ames, IA, September, 1997.

10. Hellyer, N.J., Cheng, K., and Koland, J.G. Role of protein domain interaction in p85 association with ErbB3. Growth Factor Signal Transduction Conference, Iowa State University, Ames, IA, September, 1997.

11. Koland, J.G. and Vijapurkar, U.P. Signal transduction by the ErbB3/HER3 heregulin receptor. Department of Defense Breast Cancer Research Program Meeting, Washington, DC, November, 1997.

12. Vijapurkar, U.P., Hellyer, N.J., Kratz, D.A., and Koland, J.G. Role of PI 3-kinase and MAPK pathways in heregulin-stimulated mitogenesis by the ErbB2/ErbB3 coreceptor complex. Molecular Targets for Cancer, Houston TX, October, 1998.

Presentations:

1. Koland, J.G. Signal transduction by receptors and oncogenes with tyrosine kinase activity. Hematology-Oncology Grand Rounds, University of Iowa, College of Medicine, Iowa City, IA, January, 1993.

2. Koland, J.G. Phosphorylation of the ErbB3/HER3 gene product by the EGF receptor tyrosine kinase. Translating Advances from the Bench Top to the Bed Side, University of Iowa Cancer Center, Iowa City, IA, June, 1994.

3. Koland, J.G. Growth factor receptors and oncogenes with protein tyrosine kinase activity. Dietary Phytoestrogens: Cancer Cause or Prevention, NCI, Herndon, VA, September, 1994.

4. Koland, J.G. Signal transduction by epidermal growth factor and heregulin is mediated by a kinase-deficient ErbB3 protein. Diabetes and Endocrinology Research Center Symposium, Iowa City, IA, April, 1996.

5. Koland, J.G. Signal transduction mechanisms of EGF receptor/ErbB family members. Southern Research Institute, Birmingham, AL, December, 1997.

6. Koland, J.G. Signaling mechanisms of ErbB2/ErbB3 heregulin coreceptors. Department of Molecular Biology, Vanderbilt University, Nashville, TN, August, 1998.

PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

1. John Koland   Principal Investigator
2. Deborah Kratz   Research Associate
3. Dolores Bravo   Research Associate
4. Ulka Vijapurkar   Graduate Student
5. Nathan Hellyer   Graduate Student
6. Kalyani Korabathina   Summer Undergraduate Research Program
7. Czarina Belarmino   Laboratory Assistant
8. Kim-Dung Phan   Laboratory Assistant
9. Jenny Phan   Laboratory Assistant
10. Cat Vinh   Laboratory Assistant
11. Brian Darsidan   Laboratory Assistant
12. Mikel Moore   Laboratory Assistant
13. Andrea Distelhorst   Laboratory Assistant

Epidermal Growth Factor-dependent Association of Phosphatidylinositol 3-Kinase with the *erb*B3 Gene Product*

Hong-Hee Kim, Susan L. Sierke, and John G. Koland‡

From the Department of Pharmacology, The University of Iowa, College of Medicine, Iowa City, Iowa 52242-1109

The ErbB3 protein is a member of the ErbB subfamily of receptor protein tyrosine kinases. In the present study, the mechanism by which the ErbB3 protein is phosphorylated and the signal-transducing functions of this phosphorylated protein were investigated. When phosphorylated by the epidermal growth factor receptor *in vitro*, the ErbB3 protein strongly associated with the regulatory p85 subunit and the catalytic activity of phosphatidylinositol (PI) 3-kinase. The association of PI 3-kinase with ErbB3 in human breast cancer cells was found to be correlated with the constitutive phosphorylation of ErbB3 on tyrosine residues. In MDA-MB-468 breast cancer cells in which the ErbB3 protein is not constitutively phosphorylated, stimulation with epidermal growth factor led to the phosphorylation of ErbB3 on tyrosine residues and the formation of a functional signal transduction complex involving the ErbB3 protein and PI 3-kinase. These results suggest that the ErbB3 protein can be phosphorylated on tyrosine residues by a cross-phosphorylation mechanism and that the phosphorylated ErbB3 protein can couple other growth factor receptor protein tyrosine kinases to the PI 3-kinase pathway in a manner similar to the insulin receptor substrate 1 protein.

The *erb*B3 gene product is a member of the ErbB receptor protein tyrosine kinase subfamily (1–3). Each of the receptors in this family is composed of an extracellular ligand-binding domain, a transmembrane domain, a protein tyrosine kinase domain, and a C-terminal autophosphorylation domain. A great body of evidence has implicated ErbB family members in tumorigenesis. A homolog of the epidermal growth factor (EGF)[1] receptor, v-ErbB, lacks an extracellular ligand-binding domain and has been shown to transform fibroblasts and erythroid cells and to be responsible for induction of fibrosarcomas, as well as erythroid leukemias in chicks infected by avian erythroblasto-

sis viruses (4–6). Enhanced expression of the EGF receptor and the ErbB2 protein, occurring as a result of gene amplification or mRNA overproduction, has been documented in various human cancers and is associated with poor prognosis (7–10). A consequence common to the truncation of the extracellular ligand-binding domain in v-ErbB and the overproduction of the EGF and ErbB2 receptors is an exaggerated expression of protein tyrosine kinase activity by these proteins. The v-ErbB protein tyrosine kinase has been shown to be constitutively active in the absence of any stimulant (11, 12), and constitutive activation of the ErbB2 protein kinase has been detected in NIH 3T3 cells that overexpress the protein and also in some human cancer cell lines (13–15).[2] These results indicate that the constitutive activation of the catalytic activity of these proteins may be involved in cellular transformation and in the pathogenesis of cancer. Although their exact roles in malignancy remained to be identified, overexpression of the newly identified *erb*B3 and *erb*B4 genes has been shown to occur in certain human cancer cells (16–18). Intriguingly, the ErbB3 protein has been found to be constitutively phosphorylated on tyrosine residues in a subset of breast cancer cell lines (19), and its overexpression has been associated with the presence of lymph node metastases in human breast cancers (16).

A sequence analysis of the amino acids encoded by the *erb*B3 gene revealed an overall 40–60% homology with other members of the ErbB family (1, 18), with the least similarity found in the predicted C-terminal domain of the protein. Strikingly, the kinase domain of the human ErbB3 protein has deviations at three positions invariantly conserved in all known protein tyrosine kinases. Cys-721, His-740, and Asn-815 in ErbB3 correspond to Ala, Glu, and Asp, in all other known protein tyrosine kinases, respectively (1, 20). The latter two amino acids are conserved not only in protein tyrosine kinases but also in all serine/threonine kinases identified to date. X-ray crystal structure analysis of protein kinase A indicates that the conserved Glu residue plays a key role in recognition of the phosphates of MgATP and that the conserved Asp residue functions as a catalytic base (21, 22). Furthermore, the substitution of Asn for the conserved Asp residue in the Kit and Fps proteins abolishes their kinase activities (23, 24). The presence of these amino acid deviations in the ErbB3 protein tyrosine kinase domain therefore raises the possibility that the catalytic activity of this protein might be significantly reduced relative to that of other ErbB family members.

The C-terminal autophosphorylation domains of growth factor receptor protein tyrosine kinases play an important role in the first step of the transduction of the external signals to the nucleus. Tyrosine residues in the autophosphorylation domains of these receptors become rapidly phosphorylated upon binding of their cognate ligands. The phosphorylated tyrosine residues in certain sequence contexts then interact with specific SH2

[1] The abbreviations used are: EGF, epidermal growth factor; EGFR-IC, cytoplasmic domain of the EGF receptor; EGFR-TK, protein tyrosine kinase domain of the EGF receptor; ErbB3-IC, cytoplasmic domain of the ErbB3 protein; GST, glutathione *S*-transferase; PLC-γ1, phospholipase C-γ1; GRB2, growth factor receptor-bound protein 2; IRS-1, insulin receptor substrate 1; PDGF, platelet-derived growth factor; SH2, Src homology domain 2; PI, phosphatidylinositol; PIP, phosphatidylinositol monophosphate; PI-3-P, phosphatidylinositol 3-phosphate; PI-4-P, phosphatidylinositol 4-phosphate; gPI, glycerophosphoinositol; PAGE, polyacrylamide gel electrophoresis; mAb, monoclonal antibody; HPLC, high performance liquid chromatography.

[2] H.-H. Kim, S. L. Sierke, and J. G. Koland, unpublished observation.

(src homology 2) domains (25) contained in cellular target proteins (26, 27). The C-terminal tail of the *erb*B3 gene product contains at least 9 potential tyrosine phosphorylation sites in amino acid sequence contexts that are consensus binding sites for signal-transducing proteins, including the src family members, the GRB2 protein, and the p85 subunit of PI 3-kinase (26). Notably, the amino acid sequence motif Tyr-Xaa-Xaa-Met (YXXM) for p85 binding (28) is repeated seven times. The multiple presence of the YXXM motif strongly suggests that the *erb*B3 gene product may act as an efficient recruiter of PI 3-kinase. The insulin receptor substrate 1 (IRS-1) protein is the only other known example of a protein containing several YXXM motifs and has been demonstrated to function as an adaptor between PI 3-kinase and the insulin receptor (29). PI 3-kinase is composed of an 85-kDa regulatory subunit and a 110-kDa catalytic subunit, which phosphorylates phosphatidylinositol lipids at the D-3 position of the inositol ring (30). As PI 3-kinase activity has been implicated in cellular transformation and mitogenesis (31, 32), an efficient recruitment of this enzyme by the ErbB3 protein could therefore generate transforming and mitogenic responses in the cell.

In order to constitute a functional signal transduction pathway involving PI 3-kinase, the ErbB3 protein would need to be phosphorylated on tyrosine residues. This prerequisite could be fulfilled by either autophosphorylation or a cross-phosphorylation mediated by another cellular protein tyrosine kinase. Whereas most receptor protein tyrosine kinases, including the other ErbB family members, have intrinsic autophosphorylation activity, the presence of aberrant amino acids in the kinase domain of ErbB3 makes the catalytic capability of this protein subject to question and necessitates a rigorous assessment of its enzymatic activity. Regardless of its intrinsic tyrosine kinase activity, phosphorylation of the ErbB3 protein by other protein tyrosine kinases is, however, plausible. The ErbB2 protein has been demonstrated to be phosphorylated on tyrosine residues by the EGF receptor (33, 34). To test the possibility of the cross-phosphorylation of the ErbB3 protein, we previously performed experiments with recombinant ErbB family proteins and found that the ErbB3 protein is an excellent substrate for the EGF receptor protein tyrosine kinase.[3] This result prompted our further investigations of a novel signal transduction mechanism involving the EGF receptor, the ErbB3 protein, and PI 3-kinase.

Through *in vitro* experiments, the ErbB3 protein, when phosphorylated on tyrosine residues by the EGF receptor, was shown to strongly associate with the p85 subunit and catalytic activity of PI 3-kinase. The binding of PI 3-kinase to the ErbB3 protein was confirmed by *in vivo* studies of cultured breast cancer cells in which the ErbB3 protein is present in a constitutively phosphorylated form. In subsequent studies of MDA-MB-468 breast cancer cells, in which the ErbB3 protein is not constitutively phosphorylated, the EGF-stimulated phosphorylation of the ErbB3 protein was shown. Finally, the EGF-dependent formation of a functional signal transduction complex involving ErbB3 and PI 3-kinase was demonstrated. These results led us to propose a role for ErbB3 as a diversifier of the signal transduction pathways of other ErbB family members (Fig. 9). In this paradigm the ErbB3 protein, when phosphorylated by the EGF receptor protein tyrosine kinase, efficiently recruits the PI 3-kinase component to the signal transduction pathway of the EGF receptor.

EXPERIMENTAL PROCEDURES

Materials—The entire intracellular domains of the EGF receptor (EGFR-IC) and the ErbB3 protein (ErbB3-IC) and the protein tyrosine

kinase domain of the EGF receptor (EGFR-TK) were expressed with the baculovirus/insect cell system. Details of the construction of baculovirus transfer vectors and purification of the recombinant proteins will be described elsewhere. The EGFR-IC construct incorporated the entire cytoplasmic amino acid sequence from residues 645 to 1186 of the human EGF receptor (EGFR) (35). The EGFR-TK protein did not contain the autophosphorylation domain and consisted of amino acid residues 645–972 of EGFR. The ErbB3-IC protein included the entire cytoplasmic domain of the rat ErbB3[4] protein from amino acid residues 649 to 1320. Generation of the glutathione *S*-transferase (GST) protein and a GST fusion protein incorporating the entire SH2 domain of the chicken *src* gene product has been described previously (36). The GST fusion proteins incorporating the full-length human p85α and GRB2 sequences were prepared by similar methods. The pGEX-p85 (37) and pGEX-GRB2 expression plasmids were kindly provided by Drs. Richard A. Cerione and Jeffrey E. Pessin, respectively. [γ-32P]ATP (3000 Ci/mmol) and [2-3H]inositol were acquired from DuPont-NEN. Monoclonal antibody (mAb) 291–3A and a polyclonal rabbit antiserum that both recognize the EGF receptor have been described elsewhere (38). The phosphotyrosine antibody (PY20) was obtained from Leinco Technologies, and antibodies specific for EGFR (LA1), PLC-γ1, and p85 were purchased from Upstate Biotechnology. Anti-GRB2 was obtained from Transduction Laboratories. Anti-mouse Ig and anti-rabbit Ig horseradish peroxidase conjugates and ECL detection reagents were acquired from Amersham. Normal mouse IgG and a biotinylated antibody specific for the Fc fragment of mouse IgG were acquired from Jackson ImmunoResearch Laboratories. Streptavidin-horseradish peroxidase conjugate was obtained from Zymed Laboratories. L-α-Phosphatidylinositol sodium salt was purchased from Avanti Polar-Lipids. Silica thin layer chromatography plates and a Partisil 10 SAX-HPLC column were provided by Whatman.

Generation of ErbB3-specific Polyclonal and Monoclonal Antibodies—The ErbB3-IC protein produced with the baculovirus expression system was used as an immunogen to generate a panel of mouse monoclonal antibodies that specifically recognized the cytoplasmic domain of the ErbB3 protein. The cell culture supernatants of hybridoma 2F12 and 3H72 yielded high-titer mAb preparations that immunoprecipitated both the human and rat ErbB3 proteins and also recognize both proteins in Western immunoblots. The mAb 2F12 did not significantly interact with the human EGF receptor or ErbB2 proteins.[5] The IgG in hybridoma supernatant 2F12 was purified by protein A-agarose column chromatography and used in some immunoprecipitation experiments.

A polyclonal antibody which specifically recognized the ErbB3 protein was prepared by immunization of rabbits with a recombinant C-terminal domain of the rat ErbB3 protein that was expressed as an *Escherichia coli* maltose-binding protein fusion protein (39). Serum was harvested and ErbB3-specific antibodies were affinity-purified by adsorption to an Affi-Gel-15 matrix (Bio-Rad) with an immobilized ErbB3 antigen expressed as a GST fusion protein. The antibody preparation interacted with both rat and human ErbB3 proteins and did not interact with the EGF receptor.[5]

In Vitro Binding Assays—0.5 μM EGFR-IC protein or a mixture of 0.5 μM ErbB3-IC and 0.125 μM EGFR-TK was incubated under phosphorylating conditions in buffer A (20 mM NaHEPES, 50 mM NaCl, 10% (v/v) glycerol, pH 7.4) supplemented with 15 μM ATP, 10 mM MnCl₂, and 0.1% Triton X-100 for 30 min at 22 °C. Reactions were quenched by adding NaEDTA, pH 7.4, to 45 mM, and aliquots of these reaction products corresponding to 62 pmol of EGFR-IC or ErbB3-IC were mixed with 113 pmol of each of the GST-Src, -p85, or -GRB2 fusion proteins or GST (in controls). The mixtures were incubated for 15 min on ice and then allowed to bind glutathione-agarose for an additional 15 min at 4 °C. The agarose suspensions were centrifuged for 5 s at 10,000 × g. The pellets were washed three times in 500 μl of ice-cold buffer A and then suspended in gel sample buffer. Proteins in the supernatants and the pellets were resolved by SDS-PAGE and subjected to immunoblot analysis with monoclonal antibodies 291–3A (anti-EGFR) and PY20 (anti-phosphotyrosine) and a polyclonal erbB3 antibody. For the cellular component binding assay, 10 μg of the ErbB3-IC protein in a 100-μl volume was phosphorylated in the presence or absence of ATP as described above. The phosphorylation reaction was stopped with NaEDTA and then incubated with 500 μl of 293 human embryonic kidney cell lysate for 20 min at 4 °C. ErbB3 and associated proteins were immunoprecipitated with 50 μl of the ErbB3 mAb 3H72. The immunoprecipitates were

---

[3] S. L. Sierke and J. G. Koland, unpublished observation.

[4] N. J. Hellyer, S. L. Sierke, C. H. Greaves, and J. G. Koland, manuscript in preparation.

[5] J. G. Koland, unpublished observation.

washed and suspended in 120 µl of gel sample buffer. 30-µl aliquots were subjected to SDS-PAGE and immunoblotting with anti-PLC-γ1, anti-p85, or anti-GRB2.

Culture, Lysis, and Stimulation of Cells—MDA-MB-453, MDA-MB-134, MDA-MB-468, SK-BR-3, NIH 3T3, and 293 cell lines were purchased from the American Type Culture Collection (ATCC) and maintained as recommended by ATCC. To prepare cell lysates, 70–90% confluent cultures were washed twice in ice-cold phosphate-buffered saline and incubated for 15 min on ice with a lysis buffer containing 50 mM NaHEPES, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 1% Triton X-100, 10% (v/v) glycerol, 2 µg/ml pepstatin A, 10 µg/ml aprotinin, 10 µg/ml leupeptin, pH 7.4. The whole cell lysates were centrifuged for 10 min at 10,000 × g. After protein concentration was assayed, the supernatants were either used for immunoprecipitation (see below) or directly subjected to immunoblotting. Before EGF challenge, MDA-MB-468 cells were serum starved for 16–18 h in Leibovitz's L-15 medium and then incubated with either 250 ng/ml EGF or vehicle for 3 min at 37 °C. Cells were washed immediately with phosphate-buffered saline and lysed as above.

Immunoprecipitation and Immunoblotting—Indicated amounts of ErbB3-IC and EGFR-IC recombinant proteins or cancer cell lysates were incubated with an antibody specific for ErbB3 (2F12 or 3H72), phosphotyrosine (PY20), or EGF receptor (monoclonal LA1 or polyclonal rabbit serum) for 1 h on ice. Protein G-agarose (for monoclonal antibodies) or protein A-agarose (for polyclonal antibodies) was added and rocked for an additional hour at 4 °C. The agarose matrix and associated proteins were collected by centrifuging for 10 s at 10,000 × g. The pellet was either subjected to PI 3-kinase assay procedures (see below) or washed five times in 20 mM NaHEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.1% Triton X-100, 10% (v/v) glycerol, pH 7.4, prior to immunoblot analysis. Here, the immunoprecipitate was suspended in gel sample buffer, subjected to SDS-PAGE, transferred to a polyvinylidene difluoride or nitrocellulose membrane, and immunostained with an indicated antibody. Blots were incubated with either sheep anti-mouse Ig-horseradish peroxidase or donkey anti-rabbit Ig-horseradish peroxidase and detected by ECL luminography. For GRB2 detection, two incubation steps with biotinylated anti-mouse IgG Fc and streptavidin-horseradish peroxidase conjugate replaced the incubation with secondary antibody-horseradish peroxidase conjugates.

PI 3-Kinase Assay and Identification of PIP Isomers—PI 3-kinase assays were performed as described previously (40) except that the washed immunoprecipitates were suspended in 45 µl of 10 mM Tris, pH 7.4, containing 150 mM NaCl and 5 mM EDTA and that the reaction was initiated by adding 15 µl of 107 µM ATP containing 50 µCi of [γ-^{32}P]ATP (30 Ci/mmol), 10 mM MgCl$_2$, and 45 mM NaHEPES, pH 7.8. To confirm the identity of the PI 3-kinase assay products, PIP were extracted from thin layer chromatography plates and deacylated as described previously (41). A 2-ml aliquot of each deacylated sample was mixed with [^{3}H]gPI-4-P (prepared as described below), AMP, and ADP and separated by HPLC with an anion-exchange column and a gradient of ammonium phosphate, pH 3.8. The gradient was formed from buffers A (10 mM NH$_4$H$_2$PO$_4$) and B (0.5 M NH$_4$H$_2$PO$_4$) with a dual-pump program (0% B for 5 min, to 25% B over 45 min, 25% B for 2 min, to 100% B over 5 min, 100% B for 3 min, to 0% B over 15 min). Eluate fractions collected every 0.5 min were counted in 5 ml of BudgetSolve (Research Products International) using two channels. The ^{3}H-labeled gPI-4-P isomer used as an internal standard was prepared as described previously (41). Briefly MDA-MB-468 cells were metabolically labeled with 3 µCi/ml [2-^{3}H]inositol. Cells were scraped into CH$_3$OH/HCl (100:1) after extensive washing with Leibovitz's L-15 medium, 0.1% bovine serum albumin. Lipids were extracted with CHCl$_3$, dried under nitrogen gas, resuspended in CHCl$_3$/CH$_3$OH (1:1), and separated by thin layer chromatography. The area corresponding to PI-4-P, the major cellular PIP isomer (42), was scraped and processed as were the unknowns.

RESULTS

In Vitro Association of SH2 Domain Proteins with ErbB3—In order to determine whether the tyrosine residues of ErbB3 phosphorylated by the EGF receptor constitute binding sites for signal-transducing SH2 domain proteins, *in vitro* binding assays were performed with the baculovirus-expressed cytoplasmic domain of ErbB3 (ErbB3-IC). The ErbB3-IC protein was first phosphorylated with the EGFR-TK enzyme and then allowed to bind the SH2 domain of Src, the p85 subunit of PI
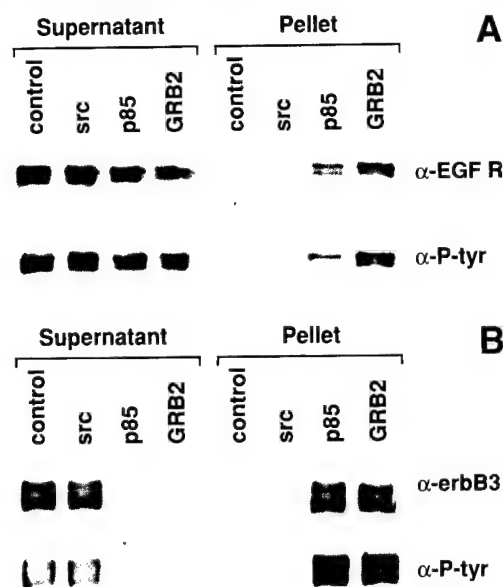

Fig. 1. **Interaction of phosphorylated ErbB3 and EGF receptor cytoplasmic domains with SH2 domain proteins.** *A*, the EGF receptor-derived EGFR-IC protein was incubated under phosphorylating conditions and then allowed to interact with SH2 domain proteins expressed as GST fusion proteins. The SH2 domain proteins tested were the c-Src SH2 domain and the full-length p85 and GRB2 proteins. The GST protein with no fused sequences was used in control experiments. Binding of the phosphorylated EGFR-IC protein to the GST fusion proteins was analyzed by glutathione-agarose precipitation, SDS-PAGE, and immunoblotting with either anti-EGFR (291–3A) or anti-phosphotyrosine (PY20). *B*, the ErbB3-IC protein was phosphorylated with the EGFR-TK enzyme (see "Experimental Procedures"), and the SH2 domain-binding properties of phosphorylated ErbB3-IC were assayed as described in *A*, except that binding was detected by immunoblotting with anti-ErbB3 (polyclonal) and anti-phosphotyrosine (PY20).

3-kinase, or the GRB2 protein each fused to GST. After extensive washing of the glutathione-agarose precipitates, both the bound and unbound proteins were analyzed by immunoblotting with anti-erbB3 and anti-phosphotyrosine. The cytoplasmic domain of the EGF receptor (EGFR-IC) was assayed in parallel to compare its ability to bind the different SH2 domain proteins. The EGFR-IC was autophosphorylated and then processed as above, except that anti-EGFR and anti-phosphotyrosine were used to detect the protein. As shown in Fig. 1, the p85 and GRB2 proteins bound the phosphorylated ErbB3 protein with high efficiency and the phosphorylated EGF receptor with lower efficiency. Under the same experimental conditions, no interaction of the ErbB3 or EGF receptor proteins with the SH2 domain of the Src protein was observed. The GST control protein also did not precipitate either the phosphorylated ErbB3 or EGF receptor protein (Fig. 1).

In similar experiments, cellular SH2 domain proteins that interacted with the phosphorylated ErbB3 protein were identified. The recombinant ErbB3-IC protein incubated with the EGFR-TK enzyme under phosphorylating or non-phosphorylating conditions was mixed with a lysate of human embryonic kidney cells 293. Cellular proteins that associated with the ErbB3 protein were then detected by immunoprecipitation and immunoblotting analyses. The p85 subunit of PI 3-kinase, PLC-γ1, and GRB2 were shown to bind ErbB3, when the ErbB3 protein was phosphorylated by the EGF receptor protein tyrosine kinase (Fig. 2). The affinity of p85 binding appeared to be higher than that of PLC-γ1 or GRB2 when the fractions of each component bound to ErbB3 were compared. Tyrosine phosphorylation of the ErbB3 protein and an equivalent immunoprecipitation of ErbB3 both in the presence and in the absence of ATP were confirmed by immunoblotting with phosphotyrosine
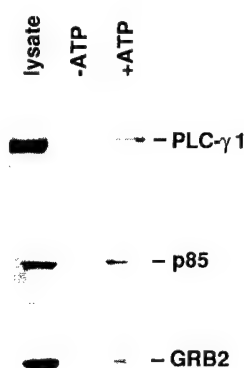
FIG. 2. **Interaction of the phosphorylated ErbB3 protein with SH2 domain proteins in cell lysates.** Samples of the ErbB3-IC protein were incubated with the EGFR-TK enzyme under phosphorylating (*+ATP*) or non-phosphorylating (*−ATP*) conditions. After incubation with the 293 cell lysate, the ErbB3-IC protein was immunoprecipitated. Cellular proteins associating with ErbB3-IC were identified by immunoblotting with PLC-γ1, p85, and GRB2 antibodies. Aliquots of the original cell lysate corresponding to ~1/20 of the immunoprecipitated volume were also analyzed and shown for comparison. Results are representative of three separate experiments.

and ErbB3 antibodies, respectively (data not shown). Repeated experiments with lysates of NIH 3T3 fibroblasts produced similar results except that much less PLC-γ1 binding was evident.

The high affinity interaction between p85 and ErbB3 observed suggested that an association of the enzymatic activity of PI 3-kinase with the phosphorylated ErbB3 protein might also occur. To examine the extent to which PI 3-kinase activity associated with the ErbB3 and EGF receptor proteins, the ErbB3-IC and EGFR-IC proteins were phosphorylated as in the previous experiments. The phosphorylated proteins were incubated with cell lysate and then immunoprecipitated with antibodies specific for the ErbB3 and EGFR proteins. Immunoprecipitates were subsequently assayed for PI kinase activity with phosphatidylinositol as the substrate (40). PIP was produced by the anti-ErbB3 immune complexes when the ErbB3 protein was phosphorylated on tyrosine residues but not by the anti-EGFR immunoprecipitates (Fig. 3A). The ErbB3-associated PI kinase activity was abolished when 0.5% Nonidet P-40 was included in the reaction (data not shown), which suggested that PI 3-kinase was responsible for the lipid product rather than PI 4-kinase (43). To confirm the identity of the PIP product, the product was extracted, deacylated, and subjected to HPLC analysis. Elution of the ^{32}P-labeled product ahead of the gPI-4-P internal standard (Fig. 3B) confirmed the identity of the PIP product as the PI-3-P isomer (30).

Association of PI 3-Kinase with the Constitutively Phosphorylated ErbB3 Protein in Breast Cancer Cells—The strong association of both the p85 subunit and the catalytic activity of PI 3-kinase with the ErbB3 protein phosphorylated *in vitro* led us to investigate whether the ErbB3 protein could constitute functional signal transduction complexes *in vivo*. Kraus and co-workers (19) have found that the ErbB3 protein is constitutively phosphorylated on tyrosine residues in a subset of the human breast cancer cell lines they examined. Using these cell lines, we studied the *in vivo* association of SH2 domain proteins with the constitutively phosphorylated ErbB3 protein. The results from immunoblotting analyses of anti-ErbB3 immunoprecipitates are shown in Fig. 4. Tyrosine phosphorylation of ErbB3 was detected in MDA-MB-453 and SK-BR-3 cells but not in MDA-MB-134 and MDA-MB-468 cells, which confirmed the results of Kraus *et al.* (19). Immunoblot analysis with a specific antibody revealed association of the p85 subunit of PI 3-kinase
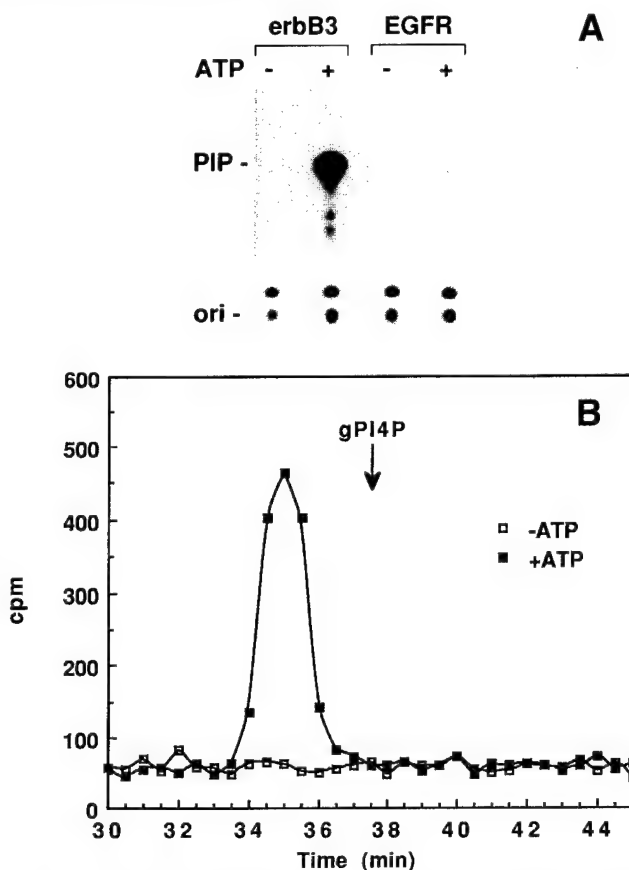

FIG. 3. **Association of PI 3-kinase activity with the phosphorylated ErbB3 protein.** 3 μg of the ErbB3-IC or EGFR-IC protein was phosphorylated in the presence or absence of ATP as described under "Experimental Procedures." After incubation with NIH 3T3 cell lysate, the recombinant proteins were immunoprecipitated with either anti-ErbB3 mAb 2F12 or polyclonal anti-EGFR, and the immunoprecipitates were assayed for PI kinase activity. *A*, thin layer chromatographic analysis of the PI kinase products is indicated. *B*, the PIP products were deacylated and analyzed by HPLC as described under "Experimental Procedures." Migration of the PIP deacylation product just ahead of the gPI-4-P standard confirms its identity as the gPI-3-P isomer.

with the ErbB3 protein in those cells in which the ErbB3 protein was phosphorylated. Although the efficiency of ErbB3 immunoprecipitation was variable as indicated in Fig. 4, repeated experiments showed that the extent to which the p85 protein associated was roughly proportional to the extent of tyrosine residue phosphorylation of ErbB3. Control immunoprecipitations with normal mouse IgG did not precipitate ErbB3 or its associated proteins (data not shown). Neither PLC-γ1 nor GRB2 was detected in the ErbB3 immunoprecipitates (Fig. 4) in contrast to the results of *in vitro* experiments (compare Figs. 1 and 2). In immunoprecipitation experiments using anti-phosphotyrosine, tyrosine phosphorylation of p85 and PLC-γ1 was detected in MDA-MB-453 cells (data not shown).

The association of the catalytic activity of PI 3-kinase with the constitutively phosphorylated ErbB3 protein was also examined. As shown in Fig. 5, significant PI 3-kinase activity was detected in immunoprecipitates from MDA-MB-453 and SK-BR-3 cells but not from MDA-MB-134 and MDA-MB-468 cells. The control immunoprecipitate with normal mouse IgG did not contain the activity of PI 3-kinase (Fig. 5A). The identity of the assay product was again confirmed as PI-3-P by HPLC analysis (Fig. 5B). These results correlated the tyrosine phosphorylation of the ErbB3 protein with its association with PI 3-kinase and suggested a constitutive activation of the lipid kinase in MDA-MB-453 and SK-BR-3 cells.
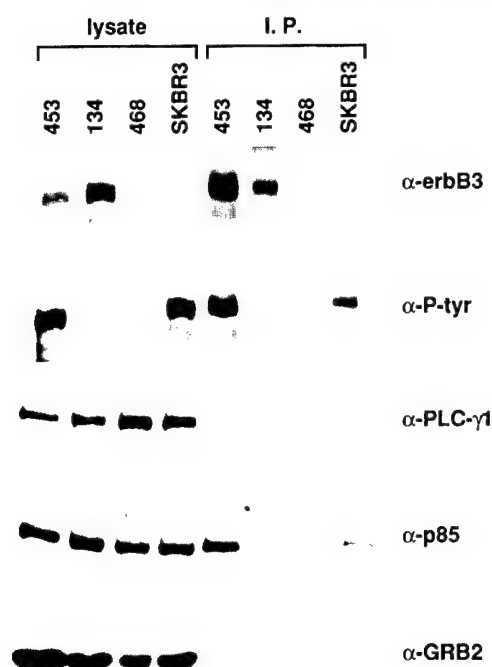
FIG. 4. **Analysis of cellular proteins associated with the phosphorylated ErbB3 protein in breast cancer cells.** MDA-MB-453, MDA-MB-134, MDA-MB-468, and SK-BR-3 cells were lysed and immunoprecipitated with anti-ErbB3 mAb 2F12 as described under "Experimental Procedures." Aliquots of immunoprecipitates were blotted with antibodies recognizing ErbB3, phosphotyrosine, PLC-γ1, p85, and GRB2. Each blot corresponds to ~1.5 mg of the centrifuged cell lysate. The lysate lanes contain 1/20 of the immunoprecipitated quantity of cellular proteins. These results are representative of three independent experiments.

EGF-stimulated Association of PI 3-Kinase with ErbB3 in MDA-MB-468 Cells—Our previous findings that the ErbB3 protein is an excellent *in vitro* substrate for the EGF receptor protein tyrosine kinase[3] and that upon phosphorylation the ErbB3 protein could interact with specific signal-transducing proteins, led us to investigate whether the EGF receptor and the ErbB3 protein could participate in a physiological signal transduction mechanism. One of the breast cancer cell lines examined, MDA-MB-468, expresses a high level of the EGF receptor and a moderate level of the ErbB3 protein (44).[2] This allowed us to examine whether the ErbB3 protein could be phosphorylated by the EGF receptor *in vivo*. MDA-MB-468 cells were serum starved, stimulated with EGF, and the ErbB3 and EGF receptor proteins were immunoprecipitated. Upon EGF stimulation, phosphorylation of the ErbB3 protein on tyrosine residues was observed, along with that of the EGF receptor (Fig. 6). Presence of equivalent amounts of ErbB3 in anti-ErbB3 immunoprecipitates from the EGF-stimulated and -unstimulated cells was confirmed by immunoblotting of the precipitates with an antibody specific for ErbB3 (data not shown). The p85 protein co-precipitated with the ErbB3 protein, but PLC-γ1 and GRB2, which are known to bind activated EGF receptors (45, 46), did not (Fig. 7). Formation of a heterodimer between the ErbB3 protein and the EGF receptor was not detected in these studies (data not shown).

To examine the possible interaction of PI 3-kinase activity with the ErbB3 protein in this system, MDA-MB-468 cell lysates were immunoprecipitated, and immunoprecipitates were assayed for PI 3-kinase activity. EGF stimulation resulted in the co-precipitation of PI 3-kinase activity with the ErbB3 protein, along with an increase of the PI 3-kinase activity in the anti-phosphotyrosine immunoprecipitate (Fig. 8A). The activ-
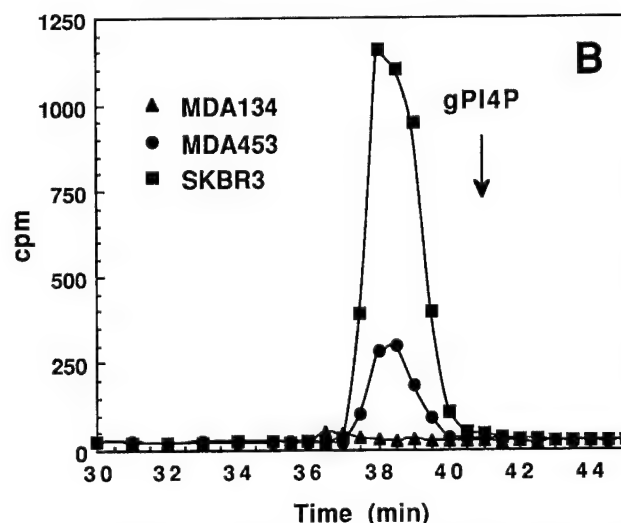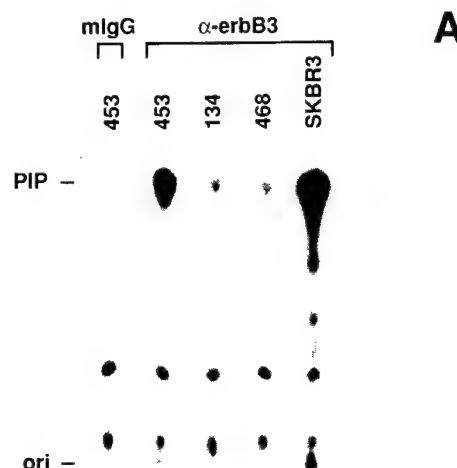
FIG. 5. **Association of PI 3-kinase activity with the phosphorylated ErbB3 protein in breast cancer cells.** *A*, 1 mg of lysate from MDA-MB-453, MDA-MB-134, or SK-BR-3 cells, or 2 mg from MDA-MB-468 cells was immunoprecipitated with anti-ErbB3 2F12. 2 mg of MDA-MB-453 cell lysate were also immunoprecipitated with normal mouse IgG as a control. The immunoprecipitates were analyzed for PI 3-kinase activity by thin layer chromatography. *B*, HPLC analysis of PIP products showed ^{32}P-labeled gPI-3-P eluting ahead of the gPI-4-P standard. Similar results were obtained in two independent experiments.

ity associated with the anti-EGF receptor precipitate was negligible and did not change upon treatment of cells with EGF (Fig. 8A). This was not due to an inability of the EGF receptor antibody to immunoprecipitate, as efficient immunoprecipitation was confirmed by anti-EGF receptor immunoblotting (data not shown). Results of HPLC analyses of glycerophosphoinositol monophosphates (gPIP) prepared from anti-ErbB3 and anti-phosphotyrosine immunoprecipitates are shown in Fig. 8, *B* and *C*, respectively. The anti-ErbB3 immune complex generated a single species of glycerophospholipid, gPI-3-P (Fig. 8B), whereas the anti-phosphotyrosine immunoprecipitate yielded a mixture of gPI-3-P and gPI-4-P isomers (Fig. 8C). The presence of PI 4-kinase in anti-phosphotyrosine column eluates and anti-EGF receptor immunoprecipitates from cells transfected with recombinant EGF receptor cDNA has previously been described by Cochet *et al.* (47). The results depicted in Figs. 6–8 indicate that the ErbB3 protein in MDA-MB-468 cells is phosphorylated on tyrosine residues in an EGF-dependent manner and that this EGF-stimulated phosphorylation of ErbB3 leads to the formation of a functional signal transduction complex involving PI 3-kinase.
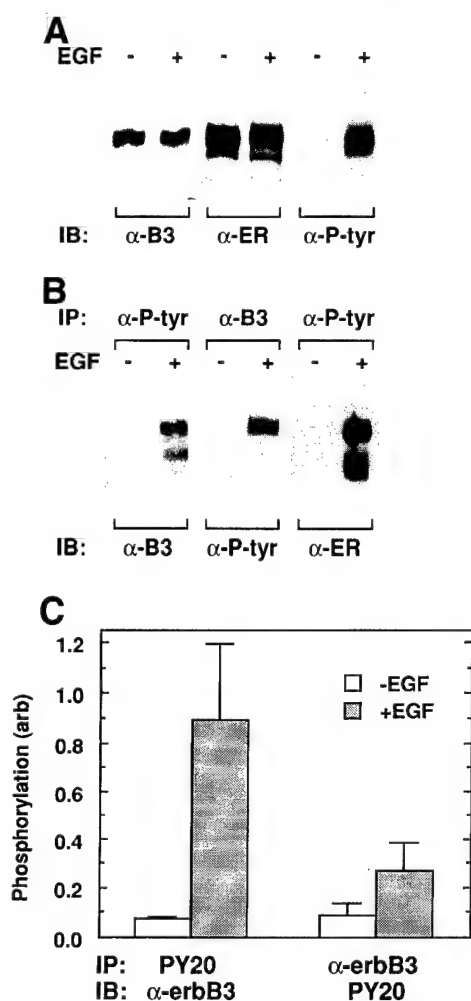
FIG. 6. **Analysis of phosphorylation of the ErbB3 protein in EGF-stimulated MDA-MB-468 cells.** *A,* serum-starved MDA-MB-468 cells were incubated with or without added EGF (250 ng/ml) for 3 min at 37 °C. Detergent lysates of the cells were prepared, and aliquots containing 100 μg of protein were resolved by SDS-PAGE. Gels were immunoblotted with either ErbB3 polyclonal antibody, EGF receptor mAb 291–3A, or phosphotyrosine-specific mAb PY20. The major bands seen in the immunoblots were in the 170–190 kDa range. *B,* cells were treated as described in *A,* and aliquots of detergent lysates containing 3 mg of protein were immunoprecipitated with either mAb PY20 or ErbB3-specific mAb 2F12. Fractions of these immunoprecipitates were resolved by SDS-PAGE and then immunoblotted with ErbB3 polyclonal antibody (two-thirds of PY20 immunoprecipitate), mAb PY20 (two-thirds of ErbB3 immunoprecipitate), or EGF receptor mAb 291–3A (one-third of PY20 immunoprecipitate) as indicated. Data of *A* and *B* are the representative results of seven separate experiments. *C,* the extent of ErbB3 phosphorylation in EGF-stimulated and control cells was assayed as described in *B* and then quantified by densitometry of the ECL luminograms. The averages and standards of deviation of triplicate experiments are shown.

FIG. 7. **Analysis of proteins associated with the ErbB3 protein in EGF-stimulated MDA-MB-468 cells.** Cells were treated as described in Fig. 6, and 4 mg of detergent lysate was immunoprecipitated with ErbB3-specific mAb 2F12. Aliquots corresponding to 1 mg of lysate protein were separated by SDS-PAGE and blotted with the indicated antibodies. These are the typical results of PLC-γ1, p85, and GRB2 blots repeated three, six, and two times, respectively.

DISCUSSION

An important paradigm in growth factor receptor signal transduction is the interaction between activated receptor protein tyrosine kinases and the SH2 domains of target proteins. The SH2 domain proteins shown to associate with ErbB family members include the Src nonreceptor protein tyrosine kinase, the p85 regulatory subunit of PI 3-kinase, PLC-γ1, and GRB2. After physical association with autophosphorylated EGF and ErbB2 receptors, PLC-γ1 becomes phosphorylated on tyrosine residues and consequently activated (48–50). Binding of GRB2 to the activated EGF receptor has been shown to initiate the Ras-mediated signal transduction pathway (46, 51). Co-precipitation of the EGF and ErbB2 receptors with both the activity
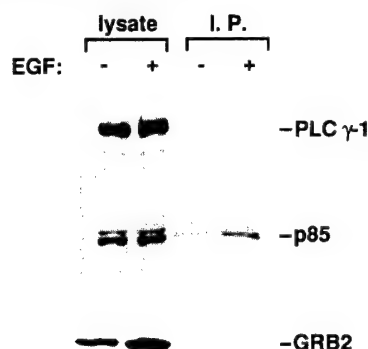
and the p85 subunit of PI 3-kinase has also been reported (52, 53). More recently, the *src* gene product has been reported to be involved in the EGF receptor signaling pathway (54).

We have examined possible interactions between these SH2 domain proteins and a newly identified member of the ErbB family, ErbB3. In two different *in vitro* binding assay systems, p85, GRB2, and PLC-γ1 were found to bind the ErbB3 protein, when ErbB3 was phosphorylated on tyrosine residues (Figs. 1 and 2). The SH2 domain of Src did not bind to the phosphorylated ErbB3 protein under the conditions of our experiments. The association of the p85 protein with ErbB3 was also observed in human breast cancer cells in which the ErbB3 protein was found to be constitutively phosphorylated on tyrosine residues (Fig. 4). The presence of PLC-γ1 and GRB2 was not detected in the anti-ErbB3 immunoprecipitates from these cancer cells, in contrast to the results of the *in vitro* experiments. The affinity of PLC-γ1 for the ErbB3 protein seemed to be lower than that of p85 (Fig. 2), and this low affinity might have attributed to this discrepancy. Fedi *et al.* (55) have generated a chimeric receptor composed of the extracellular domain of the EGF receptor and the cytoplasmic domain of the ErbB3 protein. When expressed in NIH 3T3 cells, this chimeric protein was found to associate with PI 3-kinase upon stimulation of cells with EGF. This result is in agreement with our present observations. Our failure to detect *in vivo* binding of GRB2 with ErbB3 appears to contradict the result obtained by Fedi *et al.* (Figs. 4 and 7 and Ref. 55). Although we do not exclude the possibility that the efficiency of ErbB3 immunoprecipitation was not sufficient to detect GRB2 associated with a small fraction of ErbB3, it should be noted that in the study of Fedi *et al.*, recruitment of the GRB2 protein to ErbB3 signaling complexes was indicated by an indirect immunoprecipitation analysis in which an antibody recognizing the Shc protein was employed. Alternatively, the participation of GRB2 in ErbB3 signaling may depend on cell types.

The apparently high affinity binding of p85 with ErbB3 is likely mediated by the well-characterized interaction between the SH2 domain of p85 and the Y*XX*M motifs of ErbB3. As multiple repeats of the Y*XX*M motif are present in the ErbB3 protein, simultaneous binding of several PI 3-kinase molecules to a single ErbB3 molecule is plausible. In addition to the p85 subunit, PI 3-kinase activity was also detected in immunoprecipitates of the phosphorylated ErbB3 protein (Figs. 3 and 5). Activation of PI 3-kinase activity by phosphotyrosine-containing molecules is presumably due to conformational changes in the p85 subunit and subsequent structural changes in the p110
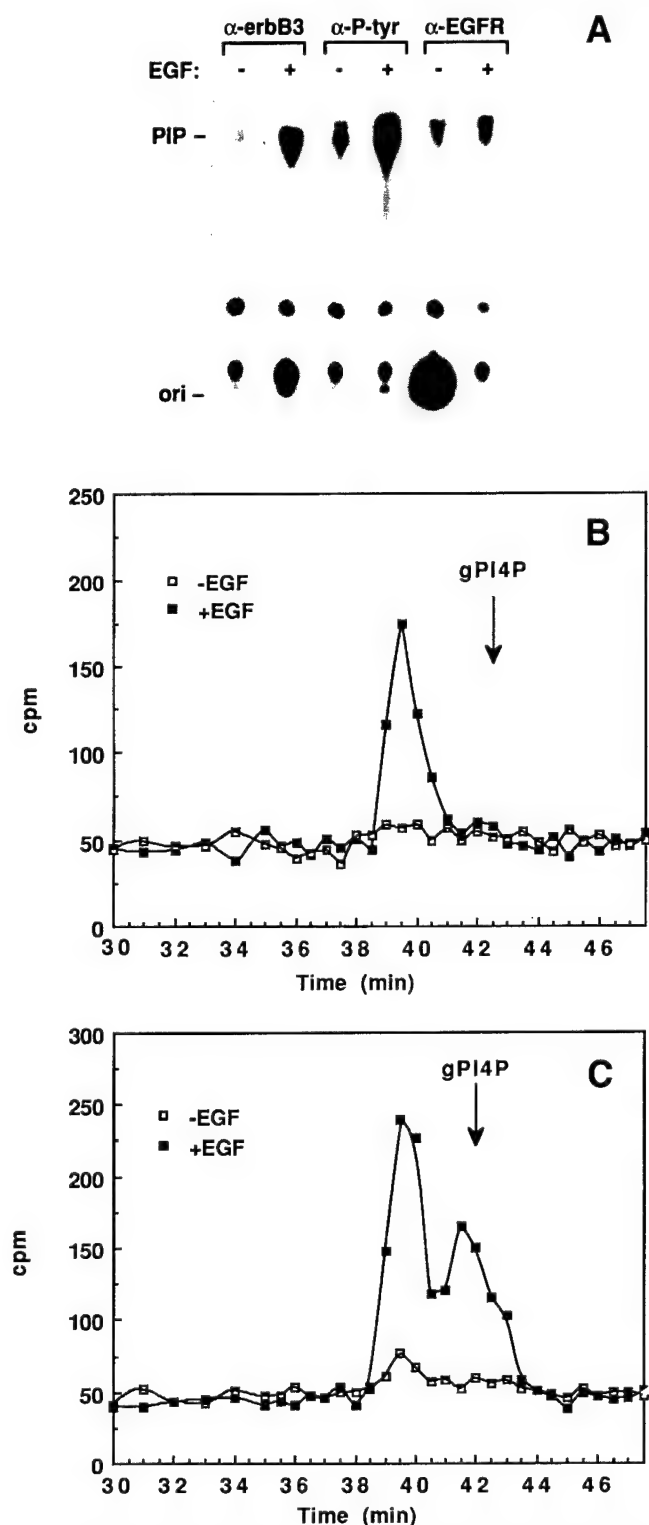
FIG. 8. **EGF-dependent association of PI 3-kinase activity with ErbB3 in MDA-MB-468 cells.** *A*, cells were treated as described in Fig. 6, and lysates were immunoprecipitated with monoclonal antibodies specific for ErbB3 (2F12), phosphotyrosine (PY20), or EGFR (LA1). The activity of PI 3-kinase in immunoprecipitates was determined as described under "Experimental Procedures." Lipid products separated by thin layer chromatography are shown. *B* and *C*, the identity of PIP lipids produced by anti-ErbB3 (*B*) and anti-phosphotyrosine (*C*) immunoprecipitates was confirmed by HPLC analysis. Similar results were obtained in a replicate experiment.

catalytic subunit. Shoelson *et al.* (56) have demonstrated changes in the structure and dynamics of the N-terminal SH2 domain of p85 upon binding of an activating phosphopeptide,

and stimulation of PI 3-kinase activity has been reported to occur by incubation with either intact phosphorylated IRS-1 or synthetic phosphopeptides containing the YXXM motif common to the IRS-1 protein, platelet-derived growth factor (PDGF) receptor, and middle T antigen (57, 58). Whereas we present here no direct evidence for the activation of PI 3-kinase upon its interaction with the ErbB3 protein, our finding that the ErbB3 protein is constitutively phosphorylated on tyrosine residues and associated with p85 in a subset of breast cancer cell lines (Figs. 4 and 5) raises the possibility that PI 3-kinase activation occurs in those cells.

The roles played by the lipid products of PI 3-kinase have only begun to be unraveled (reviewed in Ref. 30). Ling *et al.* (31) correlated the elevated level of PI-3,4-P_2 and PI-3,4,5-P_3 lipids in the cell with the transforming ability of middle T antigen variants. The importance of PI 3-kinase in cellular growth control has also been strongly indicated by the loss of the PDGF-induced mitogenic response and p85 association in cells expressing a mutated PDGF-β receptor that lacks the consensus sequence motif for p85 binding (32). Functional association of PI 3-kinase with the PDGF-β receptor also appears to be required for PDGF-dependent membrane ruffling in aortic endothelial cells (59). A role in protein sorting via vesicular transport was proposed based on the homology of the p110 catalytic subunit to the yeast Vsp34 protein (60). More recently PI 3-kinase was suggested to play an important role in T cell signaling (61). Examining the level of the cellular products of PI 3-kinase activity, the growth characteristics of breast cancer cells, and the effects of blockade of the PI 3-kinase pathway would contribute to an understanding of the possible roles of PI 3-kinase in tumorigenesis and behavior of breast cancer cells.

Stimulation of MDA-MB-468 cells with EGF led to the phosphorylation of the ErbB3 protein on tyrosine residues (Fig. 6), which suggests that ErbB3 is a cellular substrate for the EGF receptor. Although the possibility that ErbB3 becomes phosphorylated indirectly by another protein tyrosine kinase activated by the EGF receptor cannot be excluded, it is likely that the ErbB3 protein is phosphorylated directly by the EGF receptor. Our finding that the ErbB3 protein is an excellent *in vitro* substrate for the EGF receptor[3] is in support of the latter hypothesis. It has been previously shown that, in the absence of the EGF receptor, EGF does not activate the ErbB3 protein (18).

Treatment of MDA-MB-468 cells with EGF also resulted in the association of PI 3-kinase with the ErbB3 protein (Figs. 7 and 8). This EGF-dependent association of PI 3-kinase with ErbB3 is reminiscent of the insulin receptor/IRS-1 signal transduction pathway that has been characterized in much detail (29). The IRS-1 protein is phosphorylated on tyrosine residues upon activation of the insulin receptor protein tyrosine kinase, and the phosphorylated YXXM sequence motifs in IRS-1 bind with high affinity to the p85 protein, which leads to the activation of the PI 3-kinase activity of the p110 subunit (29, 57). The IRS-1 protein is therefore a key mediator in insulin receptor signaling. Like the IRS-1 protein, the ErbB3 protein also contains multiple copies of the YXXM motif (1, 2), which suggests that the ErbB3 protein may play a role in signal transduction analogous to that of IRS-1.

A cross-phosphorylation mechanism involving ErbB3 may be particularly relevant in breast cancer cells in which the concurrent overexpression of EGF receptor, ErbB2, and/or ErbB3 is common. Simultaneous overexpression of the EGF receptor and the ErbB3 protein is observed in the human breast cancer cell line MDA-MB-468 (44),[2] and high levels of both ErbB2 and ErbB3 have been detected in the MDA-MB-453, MDA-MB-361, SK-BR-3, and BT-474 breast cancer cell lines (14, 19, 44).[2]
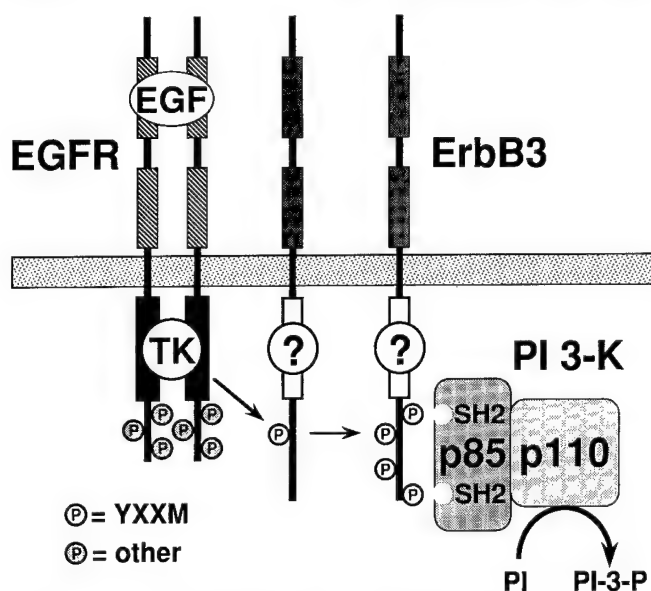
FIG. 9. **A cross-phosphorylation mechanism for signal transduction that involves the EGF receptor, the ErbB3 protein, and PI 3-kinase.** The ErbB3 protein which possesses little or no intrinsic protein tyrosine kinase activity is phosphorylated by the activated EGF receptor protein tyrosine kinase on tyrosine residues within the YXXM sequence motif. These phosphorylated tyrosine residues interact with the p85 regulatory subunit of PI 3-kinase, which could result in the activation of the p110 catalytic subunit via a conformational mechanism.

Moreover, the endogenous ErbB2 receptor protein tyrosine kinase in MDA-MB-453 and SK-BR-3 cells is apparently constitutively activated (14, 15), as is the recombinant ErbB2 protein when overexpressed in NIH 3T3 fibroblasts (13). The EGF receptor and the ErbB2 protein are not known to contain phosphorylation sites within the YXXM sequence motif. The contribution of PI 3-kinase to the signal transduction pathways of these receptors has not been conclusively demonstrated, as the association of PI 3-kinase with these receptors appears minor (53) or undetectable (62). Moreover, purified PI 3-kinase was not activated by the tyrosine-phosphorylated EGF receptor under the conditions in which the phosphorylated PDGF receptor could activate the purified enzyme (63). Our finding that PI 3-kinase activity did not associate with the EGF receptor (Figs. 3 and 8) is consistent with these observations. Phosphorylation of the ErbB3 protein by the EGF or ErbB2 receptor could, however, couple PI 3-kinase to the signal transduction pathways of these receptors, and the combined overexpression of these members of the ErbB family could diversify the signal transduction mechanisms utilized by cancer cells.

While this article was being prepared, two studies on the ligand binding properties of the ErbB3 protein have been reported (64, 65). In those studies, the ErbB3 protein, together with the ErbB2 protein, was found to constitute a high affinity binding site for heregulin β1. The heregulin-dependent phosphorylation of both ErbB2 and ErbB3 on tyrosine residues was displayed in cells expressing both proteins, whereas little phosphorylation of ErbB3 was detected in cells expressing ErbB3 alone. This heregulin-stimulated phosphorylation of ErbB3 is likely mediated by cross-phosphorylation of the ErbB3 protein by the ErbB2 receptor tyrosine kinase (64), which is consistent with our hypothesis. As depicted in Fig. 9, the ErbB3 protein may become phosphorylated on tyrosine residues by other functional tyrosine kinases, perhaps EGFR, ErbB2, and/or Src family members.

We have presented evidence for a new signal transduction mechanism involving receptors in the ErbB family. The results

of *in vitro* binding assays showed that the p85 subunit of PI 3-kinase tightly associates with ErbB3, when the ErbB3 protein is phosphorylated by the EGF receptor tyrosine kinase. EGF stimulation of MDA-MB-468 cells resulted in the association of p85 with the ErbB3 protein *in vivo*. The activity of PI 3-kinase was also linked to the tyrosine-phosphorylated ErbB3 protein, which suggested the formation of a functional signal transduction complex. We forward here a novel mechanism by which mitogenic signals could be transduced by the ErbB3 protein, which itself may have little or no intrinsic protein kinase activity. In this mechanism, the binding of EGF to the EGF receptor results in the activation of its protein tyrosine kinase activity and the phosphorylation of the ErbB3 protein on tyrosine residues. The phosphorylated ErbB3 protein then tightly binds and activates the PI 3-kinase enzyme. This novel mechanism could diversify the signal transduction pathways activated by the EGF receptor protein.

Addendum

As this manuscript was being submitted, the EGF-dependent association of the ErbB3 protein with PI 3-kinase in human A431 carcinoma cells was documented (Soltoff, S. P., Carraway, K. L., III, Prignet, S. A., Gullick, W. G., and Cantley, L. C. (1994) *Mol. Cell. Biol.* **14,** 3550–3558).

REFERENCES

1. Plowman, G. D., Whitney, G. S., Neubauer, M. G., Green, J. M., McDonald, V. L., Todaro, G. J., and Shoyab, M. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 4905–4909
2. Kraus, M. H., Issing, W., Miki, T., Popescu, N. C., and Aaronson, S. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 9193–9197
3. Fantl, W. J., Johnson, D. E., and Williams, L. T. (1993) *Annu. Rev. Biochem.* **62,** 453–481
4. Frykberg, L., Palmieri, S., Beug, H., Graf, T., Hayman, M. J., and Vennstrom, B. (1983) *Cell* **32,** 227–238
5. Yamamoto, T., Hihara, H., Nishida, T., Kawai, S., and Toyoshima, K. (1983) *Cell* **34,** 225–234
6. Maihle, N. J., and Kung, H. (1988) *Biochim. Biophys. Acta* **948,** 287–304
7. Slamon, D. J., Godolphin, W., Jones, L. A., Holt, J. A., Wong, S. G., Keith, D. E., Levin, W. J., Stuart, S. G., Udove, J., Ullrich, A., and Press, M. F. (1989) *Science* **244,** 707–712
8. Sainsbury, J. R. C., Farndon, J. R., Needham, G. K., Malcolm, A. J., and Harris, A. L. (1987) *Lancet* **1,** 1398–1402
9. Gullick, W. J. (1990) *Int. J. Cancer* **46,** 55–61
10. Gullick, W. J. (1991) *Br. Med. Bull.* **47,** 87–98
11. Kris, R. M., Lax, I., Gullick, W., Waterfield, M. D., Ullrich, A., Fridkin, M., and Schlessinger, J. (1985) *Cell* **40,** 619–625
12. Shu, H. K., Pelley, R. J., and Kung, H. J. (1991) *J. Virol.* **65,** 6173–6180
13. Lonardo, F., Di Marco, E., King, C. R., Pierce, J. H., Segatto, O., Aaronson, S. A., and Di Fiore, P. P. (1990) *New Biol.* **2,** 992–1003
14. Peles, E., Ben, L. R., Tzahar, E., Liu, N., Wen, D., and Yarden, Y. (1993) *EMBO J.* **12,** 961–971
15. Lupu, R., Colomer, R., Kannan, B., and Lippman, M. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 2287–2291
16. Lemoine, N. R., Barnes, D. M., Hollywood, D. P., Hughes, C. M., Smith, P., Dublin, E., Prigent, S. A., Gullick, W. J., and Hurst, H. C. (1992) *Br. J. Cancer* **66,** 1116–1121
17. Rajkumar, T., Goden, C., Lemoine, N. R., and Gullick, W. J. (1993) *J. Pathol.* **170,** 271–278
18. Plowman, G. D., Culouscou, J. M., Whitney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G., and Shoyab, M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 1746–1750
19. Kraus, M. H., Fedi, P., Starks, V., Muraro, R., and Aaronson, S. A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 2900–2904
20. Hanks, S. K., and Quinn, A. M. (1991) *Methods Enzymol.* **200,** 38–62
21. Knighton, D. R., Zheng, J., Eyck, L. F. T., Xuong, N., Taylor, S. S., and Sowadski, J. M. (1991) *Science* **253,** 414–420
22. Knighton, D. R., Zheng, J., Eyck, L. F. T., Ashford, V. A., Xuong, N., Taylor, S. S., and Sowadski, J. M. (1991) *Science* **253,** 407–414
23. Tan, J. C., Nocka, K., Ray, P., Traktman, P., and Besmer, P. (1990) *Science* **247,** 209–212
24. Moran, M. F., Koch, C. A., Sadowski, I., and Pawson, T. (1988) *Oncogene* **3,** 665–672
25. Sadowski, I., Stone, J. C., and Pawson, T. (1986) *Mol. Cell. Biol.* **6,** 4396–4408
26. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) *Cell* **72,** 767–778

27. Koch, C. A., Anderson, D., Moran, M. F., Ellis, C., and Pawson, T. (1991) *Science* **252,** 668–674
28. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991) *Cell* **64,** 281–302
29. Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J., and White, M. F. (1991) *Nature* **352,** 73–77
30. Varticovski, L., Harrison-Findik, D., Keeler, M. L., and Susa, M. (1994) *Biochim. Biophys. Acta* **1226,** 1–11
31. Ling, L. E., Druker, B. J., Cantley, L. C., and Roberts, T. M. (1992) *J. Virol.* **66,** 1702–1708
32. Escobedo, J. A., and Williams, L. T. (1988) *Nature* **335,** 85–87
33. Connelly, P. A., and Stern, D. F. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6054–6057
34. Goldman, R., Levy, R. B., Peles, E., and Yarden, Y. (1990) *Biochemistry* **29,** 11024–11028
35. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., Schlessinger, J., Downward, J., Mayes, E. L. V., Whittle, N., Waterfield, M. D., and Seeburg, P. H. (1984) *Nature* **309,** 418–425
36. Sierke, S. L., Longo, G. M., and Koland, J. G. (1993) *Biochem. Biophys. Res. Commun.* **191,** 45–54
37. Barfod, E. T., Zheng, Y., Kuang, W. J., Hart, M. J., Evans, T., Cerione, R. A., and Ashkenazi, A. (1993) *J. Biol. Chem.* **268,** 26059–26062
38. Koland, J. G., O'Brien, K. M., and Cerione, R. A. (1990) *Biochem. Biophys. Res. Commun.* **166,** 90–100
39. Sierke, S. L., and Koland, J. G. (1993) *Biochemistry* **32,** 10102–10108
40. White, M. F., and Backer, J. M. (1991) *Methods Enzymol.* **201,** 65–79
41. Pignataro, O. P., and Ascoli, M. (1990) *J. Biol. Chem.* **265,** 1718–1723
42. Whitman, M., Downes, C. P., Keeler, M., Keller, T., and Cantley, L. (1988) *Nature* **332,** 644–646
43. Whitman, M., Kaplan, D., Roberts, T., and Cantley, L. (1987) *Biochem. J.* **247,** 165–174
44. Kraus, M. H., Popescu, N. C., Amsbaugh, S. C., and King, C. R. (1987) *EMBO J.* **6,** 605–610
45. Margolis, B., Li, N., Koch, A., Mohammadi, M., Hurwitz, D. R., Zilberstein, A., Ullrich, A., Pawson, T., and Schlessinger, J. (1990) *EMBO J.* **9,** 4375–4380
46. Lowenstein, E. J., Daly, R. J., Batzer, A. G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E. Y., Bar-sagi, S. D., and Schlessinger, J. (1992) *Cell* **70,** 431–442
47. Cochet, C., Filhol, O., Payrastre, B., Hunter, T., and Gill, G. N. (1991) *J. Biol. Chem.* **266,** 637–644
48. Meisenhelder, J., Suh, P. -G., Rhee, S. G., and Hunter, T. (1989) *Cell* **57,** 1109–1122
49. Peles, E., Ben, L. R., Or, E., Ullrich, A., and Yarden, Y. (1991) *EMBO J.* **10,** 2077–2086
50. Nishibe, S., Wahl, M. I., Hernandez-Sotomayor, S. M. T., Tonks, N. K., Rhee, S. G., and Carpenter, G. (1990) *Science* **250,** 1253–1256
51. Schlessinger, J. (1993) *Trends Biochem. Sci.* **18,** 273–275
52. Peles, E., Lamprecht, R., Ben-Levy, R., Tzahar, E., and Yarden, Y. (1992) *J. Biol. Chem.* **267,** 12266–12274
53. Hu, P., Margolis, B., Skolnik, E. Y., Lammers, R., Ullrich, A., and Schlessinger, J. (1992) *Mol. Cell. Biol.* **12,** 981–990
54. Luttrell, D. K., Lee, A., Lansing, T. J., Crosby, R. M., Jung, K. D., Willard, D., Luther, M., Rodriguez, M., Berman, J., and Gilmer, T. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 83–87
55. Fedi, P., Pierce, J. H., Di Fiore, P. P., and Kraus, M. H. (1994) *Mol. Cell. Biol.* **14,** 492–500
56. Shoelson, S. E., Sivaraja, M., Williams, K. P., Hu, P., Schlessinger, J., and Weiss, M. A. (1993) *EMBO J.* **12,** 795–802
57. Backer, J. M., Myers, M. J., Shoelson, S. E., Chin, D. J., Sun, X. J., Miralpeix, M., Hu, P., Margolis, B., Skolnik, E. Y., Schlessinger, J., and White, M. F. (1992) *EMBO J.* **11,** 3469–3479
58. Carpenter, C. L., Auger, K. R., Chanudhuri, M., Yoakim, M., Schaffhausen, B., Shoelson, S., and Cantley, L. C. (1993) *J. Biol. Chem.* **268,** 9478–9483
59. Wennstrom, S., Siegbahn, A., Yokote, K., Arvidsson, A., Heldin, C., Mori, S., and Claesson-Welsh, L. (1994) *Oncogene* **9,** 651–660
60. Hiles, I. D., Otsu, M., Volinia, S., Fry, M. J., Gout, I., Dhand, R., Panayotou, G., Ruiz-Larrea, F., Thompson, A., Totty, N. F., Hsuan, J. J., Courtneidge, S. A., Parker, P. J., and Waterfield, M. D. (1992) *Cell* **70,** 419–429
61. Prasad, K., Janssen, O., Kapeller, R., Raab, M., Cantley, L. C., and Rudd, C. E. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 7366–7370
62. Kaplan, D. R., Whitman, M., Schaffhausen, B., Pallas, D. C., White, M., Cantley, L., and Roberts, T. M. (1987) *Cell* **50,** 1021–1029
63. Otsu, M., Hiles, I., Gout, I., Fry, M. J., Ruiz-Larrea, F., Panayotou, G., Thompson, A., Dhand, R., Hsuan, J., Totty, N., Smith, A. D., Morgan, S. J., Courtneidge, S. A., Parker, P. J., and Waterfield, M. D. (1991) *Cell* **65,** 92–104
64. Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., III (1994) *J. Biol. Chem.* **269,** 14661–14665
65. Carraway, K. L., III, Sliwkowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C., and Cerione, R. A. (1994) *J. Biol. Chem.* **269,** 14303–14306

Heregulin-stimulated Signaling in Rat Pheochromocytoma Cells

EVIDENCE FOR ErbB3 INTERACTIONS WITH Neu/ErbB2 AND p85*

Daniel C. Gamett‡, Tricia Greene‡, Allison R. Wagreich‡, Hong-Hee Kim§, John G. Koland§, and Richard A. Cerione‡¶

From the ‡Department of Pharmacology, Cornell University, Ithaca, New York 14853-6401 and the §Department of Pharmacology, University of Iowa, College of Medicine, Iowa City, Iowa 52242-1109

We have reported that overexpression of Neu leads to heregulin-stimulated neurite outgrowth and the tyrosine-phosphorylation of Neu and other cellular proteins in PC12 cells. Considering that Neu/ErbB2 alone is not able to functionally couple to heregulin, we looked for the possible involvement of ErbB3 in these neurite outgrowth and tyrosine phosphorylation responses. We found that heregulin stimulates the tyrosine phosphorylation of endogenous ErbB3 protein in PC12 cells and that this phosphorylation, like that of Neu, is greatly enhanced in cells that overexpress Neu. Furthermore, overexpression of ErbB3 in PC12 cells led to heregulin-stimulated neurite extension. In addition to becoming tyrosine-phosphorylated, Neu/ErbB2 and ErbB3 associate with each other, and each associates with the 85-kDa regulatory subunit (p85) of phosphatidylinositol 3-kinase in a heregulin-dependent manner. Thus, Neu/ErbB2 and ErbB3 appear to cooperate to mediate the heregulin signal in PC12 cells. Like heregulin, epidermal growth factor (EGF) also stimulates the tyrosine phosphorylation of both Neu and ErbB3. However, there are clear differences between the EGF- and heregulin-stimulated phosphorylations of ErbB3. In the heregulin response, two tyrosine-phosphorylated forms of ErbB3 are detected. Of these, only the more quickly migrating form (on SDS-polyacrylamide gel electrophoresis) is found to be associated with Neu, whereas the other, more slowly migrating form is uniquely capable of forming stable complexes with p85. In the EGF response, at least two tyrosine-phosphorylated forms of ErbB3 are detected, but these phosphoproteins have distinctly lower apparent molecular weights compared with the heregulin-stimulated ErbB3 phosphoproteins and do not complex with p85. Thus the formation of a stable ErbB3-p85 complex in PC12 cells is a unique outcome of heregulin signaling that correlates with the differences in cell morphology induced by the activated EGF receptor and the Neu tyrosine kinase.

The subclass 1 receptor tyrosine kinases include the epidermal growth factor (EGF)[1] receptor, the Neu/ErbB2 tyrosine kinase, and the ErbB3 and ErbB4 proteins. This receptor family has received a great deal of attention because of the sus-pected involvement of different members of the family in the development of human cancers. This has especially been true for Neu/ErbB2. The rat Neu tyrosine kinase was first identified in rat neuroblastomas induced by the chemical mutagenesis of rat embryos (1). More recently, the human homolog of the rat Neu protein, commonly designated ErbB2 (or Her2), has been implicated in the development of human breast and cervical cancers (2).

It is interesting that a variety of studies also have implicated the involvement of receptor tyrosine kinases in developmental processes; the most well known examples being in the development of the compound eye in *Drosophila* (3) and in vulval development in *Caenorhabditis elegans* (4). In the case of Neu/ErbB2, it has been suspected that this tyrosine kinase may play a role in neuronal development, because a putative activator/growth factor for Neu/ErbB2, called heregulin (5) or the Neu differentiation factor (6), is identical to glial growth factor (7) and acetylcholine receptor-inducing activity (8). Along these lines, we recently have shown that the expression of a transforming version of the Neu tyrosine kinase (where the valine residue at position 664 within the transmembranal domain has been changed to a glutamic acid) is capable of stimulating neurite extension in rat pheochromocytoma (PC12) cells (9). Moreover, we have found that the addition of heregulin to PC12 cells that overexpress the normal Neu tyrosine kinase also elicits neurite extension. These results then highlight two important points. The first is that activation of the Neu tyrosine kinase, either by a point mutation or by the addition of its putative activating ligand, elicits a cellular morphology that is similar to that induced by the nerve growth factor receptor (*i.e.* the Trk tyrosine kinase) but distinct from the cellular effects elicited by the more similar EGF receptor. Thus, PC12 cells offer an excellent model system for distinguishing the specific features of the signaling pathways initiated by Neu/ErbB2 *versus* the EGF receptor.

The second major implication from the original studies of Neu effects in PC12 cells is the fact that the addition of heregulin leads to the apparent activation of the Neu/ErbB2 tyrosine kinase in these cells. This finding indicates that PC12 cells must contain the necessary components for the stimulatory regulation of Neu. This is an important point because we (10, 11) and others (12, 13) have shown that Neu/ErbB2 alone is not able to functionally couple to heregulin. Rather, it appears that the actual receptors for heregulin are the ErbB3 and ErbB4 proteins, suggesting that heregulin-stimulated heterodimer formation between Neu/ErbB2 and either ErbB3 or ErbB4 is necessary to stimulate Neu/ErbB2 tyrosine kinase activity. In the case of ErbB3, the need for coupling to Neu/ErbB2 is most clear, because ErbB3 by itself has little or no intrinsic tyrosine kinase activity (14).

In the present studies, we set out to expand upon these two

implications. Specifically, we wanted to determine if the ErbB3 protein participated with Neu/ErbB2 in eliciting neurite extension in PC12 cells, and we set out to identify differences between the signaling cascades initiated by heregulin and those stimulated by EGF. We found that overexpression of either Neu/ErbB2 or ErbB3 renders PC12 cells biochemically and morphologically responsive to heregulin. Neu/ErbB2 and ErbB3 associate with each other, become tyrosine-phosphorylated, and associate with the p85 regulatory subunit of phosphatidylinositol 3-kinase after heregulin treatment. The events triggered by heregulin are distinguished from those initiated by EGF by the formation of a tyrosine-phosphorylated ErbB3 species with a retarded electrophoretic mobility and unique ability to complex with p85.

MATERIALS AND METHODS

Cells, Growth Factors, and Antibodies—PC12 cells (obtained from Dr. M. Chao, Cornell University Medical School) were propagated in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated horse serum and 5% fetal bovine serum. Anti-phosphotyrosine antibody (PY20) was from Transduction Laboratories, anti-c-Neu antibody-3 was from Oncogene Science Inc. (Manhasset, NY), and anti-ErbB4 was from Santa Cruz Biotechnology. Anti-p85 antibody was a generous gift from Drs. L. Cantley and C. Carpenter (Harvard Medical School). Production and use of the 2F12 anti-ErbB3 monoclonal antibody (Neo Markers, Fremont, CA) have been described elsewhere (15). Recombinant heregulin (rHRG-$\beta 1_{177-241}$) was provided by by Dr. Mark Sliwkowski, Genentech, Inc.

Transfections—The cDNA for bovine ErbB3 (10) was subcloned into pcDNAI/neo (Invitrogen) and introduced into PC12 cells using Lipofectin (Life Technologies, Inc.) according to the supplier's protocol. Stably transfected cells were selected in 400 μg/ml (active) antibiotic G418 (Geneticin, Life Technologies, Inc.).

Growth Factor Treatment, Immunoprecipitation, and Western Blot Analysis—Cells were grown to near confluence in Dulbecco's modified Eagle's medium plus 10% horse serum and 5% fetal bovine serum on 150-mm tissue culture dishes. 16–20 h prior to growth factor treatment, media were changed to Dulbecco's modified Eagle's medium with 0.1% fetal bovine serum. Cells were then removed from the dishes by trituration in Hanks' balanced salt solution without calcium or magnesium, pelleted (500 rpm for 5 min), and resuspended in Dulbecco's modified Eagle's medium without serum. Cells were dispensed at $1.0 \times 10^7/1.5$ ml/microfuge tube. Heregulin (20 nM) or EGF (100 ng/ml) was added, and the cells were incubated at 37 °C for 5 min. The treatment was ended by plunging the microfuge tubes containing the cells into ice followed immediately by centrifugation to pellet the cells and aspiration of the factor-containing supernatants. The cell pellets were then lysed in Tris-buffered saline with 1% Nonidet P-40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 mM EGTA, 40 mM sodium fluoride, 100 μM ammonium molybdate, and 1 mM sodium orthovanadate. The lysates were incubated for 15 min on ice, after which insoluble materials were pelleted in a microfuge (10 min). Each supernatant was then incubated for 2 h on ice with 30 μl of a 50% suspension of protein A-Sepharose (Sigma) and the indicated antibodies. The immune complexes were pelleted, boiled in 40 μl of Laemmli sample buffer, fractionated by SDS-polyacrylamide gel electrophoresis (8% acrylamide), and transferred to nitrocellulose. The blots were blocked in 3% bovine serum albumin in Tris-buffered saline plus 0.05% Tween 20 for 1 h at room temperature and incubated overnight at 4 °C in primary antibody as indicated. Blots were analyzed by ECL (Amersham).

RESULTS

Overexpression of the Rat Neu Tyrosine Kinase in PC12 Cells—The overexpression of the normal rat Neu tyrosine kinase in PC12 cells could be visualized by immunoprecipitation and Western blot analysis using a specific anti-Neu monoclonal antibody (Fig. 1A). Comparison of *lanes 1* and *2* (parental PC12 cells) and *lanes 5* and *6* (PC12 cells infected with the cDNA for the normal Neu tyrosine kinase; designated PC12/NeuN) in Fig. 1 indicates that although parental PC12 cells express Neu, a significantly stronger signal is obtained when Western blotting anti-Neu immunoprecipitates from PC12/NeuN cells. This signal is not affected by a 5-min incubation with heregulin in
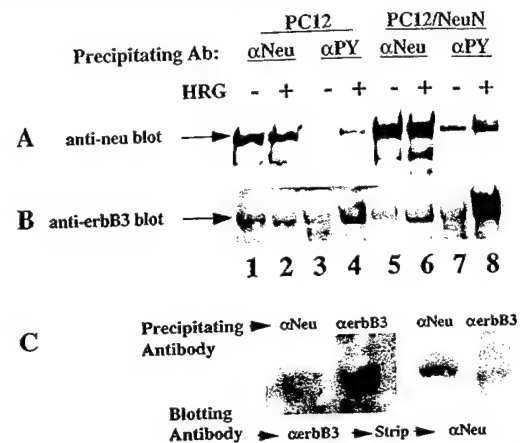

FIG. 1. **Heregulin-stimulated tyrosine phosphorylation of Neu/ErbB2 and ErbB3 and specificity of anti-ErbB3 antibody (Ab).** *A and B*, parental PC12 or PC12/NeuN (line I77.2, Ref. 9) cells (2×10^7 each) were incubated for 5 min with (+) or without (−) 10 nM heregulin (*HRG*) in serum-free medium, and cell lysates were prepared as described under "Materials and Methods." The lysates were divided equally for immunoprecipitation with antibodies against Neu (*αNeu*) or phosphotyrosine (*αPY*). *A*, the precipitates were analyzed by immunoblotting with antibody against Neu/ErbB2. *B*, a separate experiment in which the precipitates were analyzed by immunoblotting with the 2F12 monoclonal antibody against ErbB3. *C*, a lysate of PC12/NeuN cells was divided equally for immunoprecipitation with anti-Neu (*αNeu*) or anti-ErbB3 (*αErbB3*) antibodies. The precipitates were first analyzed by immunoblotting anti-ErbB3, and then the blot was stripped and reprobed with anti-Neu.

either the parental PC12 cells or the Neu transfectants.

Lanes 3 and *4* in Fig. 1A also show that Neu is tyrosine-phosphorylated in a heregulin-stimulated manner in parental PC12 cells. As expected, the tyrosine phosphorylation of Neu is stronger in Neu transfectants (Fig. 1A, *lanes 7* and *8*), and in fact a doublet is detectable for Neu transfectants treated with heregulin.

Given that we previously had shown that the ErbB3 protein, by binding heregulin, enabled Neu to become responsive to heregulin (10), we examined whether the addition of heregulin to PC12 cells stimulated the formation of a complex between Neu/ErbB2 and ErbB3. We approached this in a separate experiment using a monoclonal antibody that is highly specific for ErbB3 and does not cross-react with the rat Neu protein (monoclonal 2F12, Ref. 15; see also Fig. 1C) as the first probe (Fig. 1B). In the parental PC12 cells, we had a difficult time detecting ErbB3 above background and have been unable to draw any conclusions regarding a heregulin-stimulated ErbB3-Neu complex. However, in cells that overexpress Neu, we can clearly detect ErbB3 in the anti-Neu precipitated pellets, and this appears to be a heregulin-stimulated event (see Fig. 1B, *lanes 5* and *6*). The fact that substantially more of this heregulin-stimulated ErbB3-Neu complex is found in the PC12/NeuN cells compared with the parental PC12 cells suggests that the affinity of Neu for ErbB3 is relatively weak, such that sufficient complex formation can only be detected by immunoprecipitation under conditions where the amount of one or the other of these proteins is relatively high (also see below).

The ErbB3 protein can be clearly detected in anti-phosphotyrosine immunoprecipitates (Fig. 1B, *lanes 4* and *8*). Again, the presence of ErbB3 in these pellets is entirely dependent upon the treatment of cells with heregulin, and the amount of ErbB3 is significantly increased in the PC12/NeuN cells compared with parental PC12 cells. Note that the broad ErbB3 band detected in anti-phosphotyrosine precipitates (Fig. 1B, *lane 8*) appears to contain a component with lesser electrophoretic mobility than that found in the anti-Neu precipitates
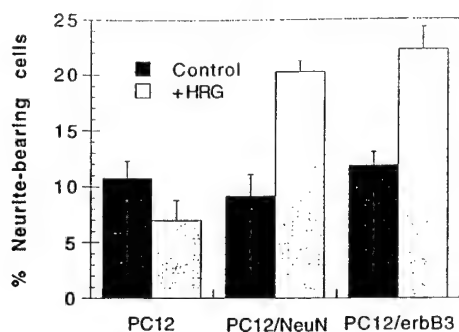
FIG. 2. **Heregulin-stimulated neurite outgrowth in PC12/ErbB3 (C59.3) cells.** Cells were incubated in 2 nM heregulin (*HRG*) in 0.5% fetal bovine serum for 3 days. Neurites were scored as described by Gamett and Cerione (9).

FIG. 3, **Heregulin stimulation of tyrosine phosphorylation of Neu and ErbB3 in parental PC12 and PC12/ErbB3 cells.** *A*, both immunoprecipitation and immunoblotting were done with anti-phosphotyrosine (*αPY*) antibodies. *B*, the cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies, and the precipitates were analyzed by immunoblotting with anti-Neu antibody (*αNeu*). *C*, the cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies, and the precipitates were analyzed by immunoblotting with the 2F12 monoclonal antibody against ErbB3 (*αErbB3*). *HRG*, heregulin.

FIG. 4. **Anti-ErbB3 immunoblots showing association of different forms of ErbB3 protein with Neu and p85.** *A*, lysates from PC12/NeuN (A1.3) were immunoprecipitated with anti-Neu (*αNeu*) (*lanes 1* and *2*), and the supernatants after this precipitation were immunoprecipitated with anti-phosphotyrosine (*αPY*) (*lanes 3* and *4*). *B*, the lysate of heregulin-stimulated cells was divided equally for immunoprecipitation with antibodies against phosphotyrosine or p85 (*αp85*). The *arrow* points to the more slowly migrating form of ErbB3 discussed in the text. *IP Ab*, immunoprecipitation antibody; *HRG*, heregulin.

(this will be considered further below). It should also be noted that although the ErbB4 protein can be detected by Western blotting anti-phosphotyrosine precipitates with a specific anti-ErbB4 antibody, the apparent tyrosine phosphorylation of ErbB4 is not stimulated by heregulin in PC12 cells (data not shown).

Overexpression of ErbB3 in PC12 Cells—Overall, the data presented in Fig. 1 indicate that the ErbB3 protein interacts with Neu in a heregulin-dependent manner and that ErbB3 either is tyrosine-phosphorylated or associates with a tyrosine-phosphorylated protein in a heregulin- and Neu-dependent manner. One simple model consistent with these findings would be that heregulin stimulates the formation of a Neu-ErbB3 heterodimer, which in turn leads to the activation of the Neu tyrosine kinase and to the tyrosine phosphorylation of the ErbB3 protein. Presumably, the formation of this heterodimer would then initiate signaling pathways that account for the heregulin-stimulated neurite extension observed in PC12 cells (9). If this were in fact the case, we would predict that overexpression of ErbB3 would facilitate heregulin-stimulated neurite extension, as was observed upon overexpression of the normal Neu protein. In order to investigate this possibility, lipofection of the bovine *erbB3* cDNA was performed to generate stable PC12 cell transfectants that overexpress the ErbB3 protein. As shown in Fig. 2, PC12 cells overexpressing ErbB3 (PC12/ErbB3, clone C59.3) showed neurite extension that was stimulated by heregulin, similar to the response observed with PC12/NeuN cells.

Also similar to PC12/NeuN cells is the pattern of ~180-kDa proteins precipitated by the anti-phosphotyrosine antibody in a heregulin-dependent manner from PC12/ErbB3 cells (Fig. 3A). Western blotting with specific monoclonal antibodies indicates that these proteins include both Neu and ErbB3 (Fig. 3, *B* and *C*). In the case of Neu (Fig. 3*B*, *lane 4*), a doublet is detectable in the Western blot (also see Fig. 1*A*, *lane 8*), suggesting that there are multiple heregulin-stimulated phosphorylation sites. For ErbB3, we frequently detect a doublet in anti-phosphotyrosine immunoprecipitates of heregulin-stimulated cells, (*e.g.* Fig. 4); the more quickly migrating band was barely visible, however, in the experiment shown in Fig. 3*C*. The predominant ErbB3 band detected in anti-phosphotyrosine immunoprecipitates migrated more slowly than either band detected upon Western blotting these precipitates with the anti-Neu antibody. Taken together, these results suggest that both the Neu and ErbB3 proteins are tyrosine-phosphorylated in a heregulin-dependent manner in the ErbB3 transfectants and that a phosphorylated ErbB3 protein has distinctly lesser mobility than either of the phosphorylated forms of the Neu protein.

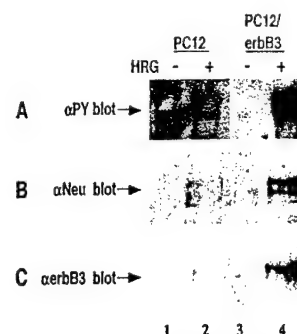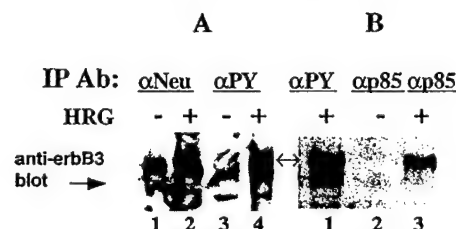*Interactions of Distinct Forms of ErbB3 with Neu and the*

85-kDa Regulatory Subunit (p85) of the Phosphatidylinositol 3-Kinase in PC12 Cells—Based on considerations of consensus sequences (16, 17), it has been predicted that the phosphorylated ErbB3 protein would bind to the 85-kDa regulatory subunit of the phosphatidylinositol 3-kinase. Indeed, binding of the ErbB3 cytoplasmic domain to p85 has been demonstrated following EGF stimulation of fibroblasts expressing an EGF receptor-ErbB3 chimera (18, 19) and ErbB3 has been shown to interact with p85 following EGF stimulation of MDA-MB-468 breast cancer cells (15) and A431 cells (20). Having seen heregulin-dependent tyrosine phosphorylation of ErbB3 in PC12 cells, we wanted to examine whether this would also lead to the interaction of ErbB3 and p85. Furthermore, the previous results (Figs. 1 and 3) had shown the existence of two or more tyrosine-phosphorylated species of ErbB3 in heregulin-treated cells, whereas only a single ErbB3 band appeared to associate with Neu, thus raising the possibility of distinct roles for the different forms of ErbB3. These questions were addressed by the experiments shown in Fig. 4. *Lanes 1* and *2* in Fig. 4*A* show the heregulin-dependent co-precipitation of ErbB3 in anti-Neu immunoprecipitates from lysates of PC12/NeuN cells. The supernatants remaining after anti-Neu precipitation were found to contain an additional, slower mobility ErbB3 band (see Fig. 4*A*, *arrow*, *lane 4*) precipitable by anti-phosphotyrosine. The appearance of this band is absolutely dependent on the addition of heregulin. These results suggest that although Neu and ErbB3 can be co-precipitated from PC12 cells overexpressing Neu, and while ErbB3 may be tyrosine-phosphorylated within this complex, there is a second phosphorylated form of
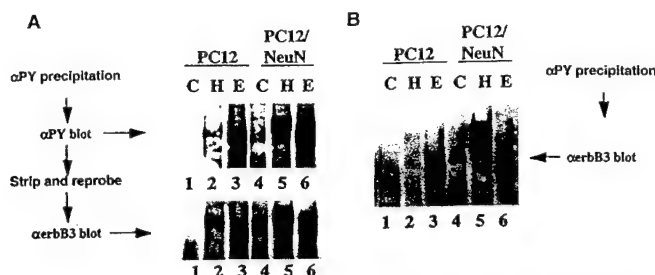
FIG. 5. **Comparison of heregulin- and EGF-stimulated tyrosine phosphorylation of ErbB3.** *A*, PC12 or PC12/NeuN (I77.2) cells were stimulated for 5 min with 10 nM heregulin or 100 ng/ml EGF (lanes marked *H* and *E*, respectively; untreated controls are marked *C*). Cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies (*αPY*), and the precipitates were analyzed by immunoblotting with anti-phosphotyrosine antibodies (*top*). Then the blot was reprobed with anti-ErbB3 (*αErbB3, bottom*). *B*, PC12 or PC12/NeuN (I77.2) cells were treated as described in *A*, then cell lysates were immunoprecipitated with anti-phosphotyrosine antibodies, and the precipitates were analyzed by immunoblotting with anti-ErbB3 antibody.

FIG. 6. **Comparison of heregulin- and EGF-stimulated complex formation between ErbB3 and p85.** PC12 and PC12/NeuN (I77.2) cells were stimulated with heregulin or EGF as described in the legend for Fig. 5. Cell lysates were immunoprecipitated with anti-p85 antibodies (*αp85*), and the supernatants were then immunoprecipitated with anti-phosphotyrosine antibodies (*αPY*). Both the anti-p85 (*top*) and anti-phosphotyrosine (*bottom*) precipitates were analyzed by immunoblotting with ErbB3 antibodies (*αErbB3*).

ErbB3 that migrates with slower mobility and is not complexed with Neu (although the generation of this ErbB3 species is dependent on heregulin and is enhanced by overexpression of Neu).

Interestingly, we find that it is this ErbB3 species that can be co-precipitated with an anti-p85 antibody (Fig. 4B). Specifically, *lane 1* in Fig. 4B shows the ErbB3 doublet obtained when Western blotting an anti-phosphotyrosine precipitate from PC12 cells overexpressing Neu, and *lanes 2* and *3* show a similar experiment using a specific anti-p85 antibody to precipitate ErbB3. In direct comparisons, we consistently find that the form of ErbB3 that complexes with p85 has an identical mobility to the upper ErbB3 band detected in anti-phosphotyrosine immunoprecipitates. Again, the ability to co-precipitate ErbB3 and p85 is strictly dependent on heregulin addition to the PC12 cells. These results are consistent with a model where heregulin stimulates the formation of a Neu-ErbB3 complex and the multiple phosphorylation of ErbB3 (see "Discussion"). One phosphorylated form of ErbB3 is able to remain complexed with Neu, whereas a second phosphorylated form, which migrates with a slower mobility on SDS-polyacrylamide gel electrophoresis, dissociates from Neu and is able to form a complex with p85.

Comparisons of the Effects of Heregulin and EGF on the Tyrosine Phosphorylation of ErbB3 and the Interactions of ErbB3 with p85—Given that in other cell systems it has been shown that the EGF receptor can couple functionally to ErbB3 and p85 (15, 20), we compared the abilities of EGF and heregulin to stimulate the phosphorylation of ErbB3 and its complex formation with p85. The *upper panel* in Fig. 5A compares the results from Western blot analyses (using an anti-phosphotyrosine antibody) where tyrosine-phosphorylated proteins were immunoprecipitated from parental PC12 cells and PC12/NeuN cells, in both the presence and the absence of treatment with heregulin or EGF. The predominant bands observed in these experiments are at ∼180 kDa, which is the expected mobility for the EGF receptor and the related Neu and ErbB3 proteins. The EGF-stimulated phosphorylation signals in the parental PC12 cells and in PC12/NeuN cells appeared to be equivalent (compare *lanes 3* and *6* in the *upper panel* of Fig. 5A), whereas, as indicated earlier (*e.g.* Fig. 1), the heregulin response in PC12/NeuN cells was enhanced relative to the parental PC12 cells (compare *lanes 2* and *5* in the *upper panel* of Fig. 5A). The results obtained in PC12 cells overexpressing Neu also clearly show that the mobilities of the phosphoprotein bands in the ∼180-kDa region differ for heregulin-treated cells compared
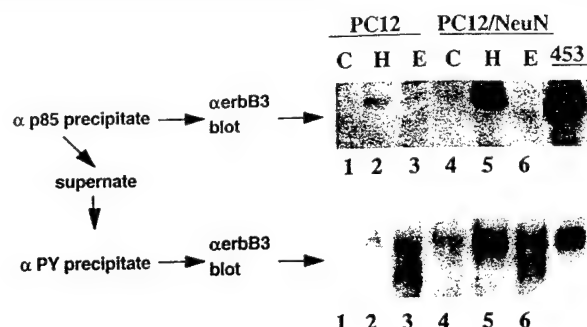
with EGF-treated cells. Heregulin treatment results in phosphoprotein bands that have less mobility than those detected following EGF-stimulation (compare *lanes 5* and *6* in the *upper panel* of Fig. 5A). These results suggest either that different proteins are being tyrosine-phosphorylated upon the addition of EGF *versus* heregulin and/or that different sites are being phosphorylated on the same proteins.

When the same anti-phosphotyrosine precipitates are reblotted with an anti-ErbB3 antibody, the overall profile obtained is very similar to that observed in the anti-phosphotyrosine blot (Fig. 5A, *lower panel*). This similarity argues that both heregulin and EGF stimulate the tyrosine phosphorylation of ErbB3 and that the stimulatory effects of heregulin but not EGF are greatly enhanced in PC12 cells overexpressing Neu. In order to be certain that these results were not artifactual due to incomplete stripping of anti-phosphotyrosine antibody prior to reprobing, we repeated the experiment and blotted first with anti-ErbB3 (Fig. 5B). Again, the electrophoretic mobilities of the ErbB3 bands are clearly different for heregulin and EGF treatment (compare *lanes 5* and *6* of Fig. 5B). The heregulin-stimulated cells showed the two forms of ErbB3 described above (Fig. 4). The faster mobility ErbB3 band observed upon heregulin treatment co-migrated with the slower mobility EGF-stimulated ErbB3 band. These results suggest that both the EGF receptor and Neu can elicit a common phosphorylation event within the ErbB3 protein and that this phosphorylated ErbB3 species (when generated by heregulin treatment) can remain complexed with Neu. There also is a broad ErbB3 band detected in EGF-treated cells that has a greater mobility than any of the bands detected in heregulin-treated cells.

The experiment shown in Fig. 6 tested for the formation of stable complexes between the various phosphorylated forms of ErbB3 seen in heregulin- and EGF-stimulated cells with p85. For this, lysates of parental PC12 cells or PC12/NeuN cells were first immunoprecipitated with an anti-p85 antibody, and the precipitates were blotted with the anti-ErbB3 antibody (Fig. 6, *upper panel*). The supernatants remaining after the anti-p85 precipitation were then immunoprecipited with anti-phosphotyrosine antibodies, and these precipitates were also analyzed by blotting with anti-ErbB3 (Fig. 6, *lower panel*). As shown in the *upper panel*, we only detect a p85-ErbB3 complex in heregulin-treated cells (Fig. 6, *lanes 2* and *5*) and not in EGF-treated cells (Fig. 6, *lanes 3* and *6*), and this complex is strongly stimulated in PC12 cells overexpressing Neu (compare *lanes 2* and *5* of Fig. 6). Again, the mobility of the ErbB3 band that co-precipitates with p85 is identical to the slower mobility ErbB3 band detected in anti-phosphotyrosine immunoprecipi-

tates. When examining the lysates that remain behind following the immunoprecipitation with anti-p85, we see both heregulin- and EGF-stimulated phosphoprotein bands in the ~180-kDa region. Thus, whereas EGF stimulates the phosphorylation of ErbB3 (as indicated in Fig. 5), this phosphorylated ErbB3 species is not able to form a stable complex with p85. As will be discussed further below, these results then point to a model where both the EGF-activated EGF receptor and the heregulin-activated Neu tyrosine kinase can phosphorylate ErbB3 in a distinct manner and that only ErbB3 proteins that have been phosphorylated by Neu can go on to form a stable complex with p85 in PC12 cells.

DISCUSSION

It originally was reported that heregulin, a 144-kDa transmembranal glycoprotein with an EGF-like region within its extracellular domain, was the ligand/growth factor for Neu/ErbB2 (5). However, recent studies have indicated that the true receptors for heregulin are the ErbB3 and ErbB4 proteins (10, 12) and that it is heterodimer formation between Neu and ErbB3 (or Neu and ErbB4) that enables the Neu tyrosine kinase activity to be stimulated by heregulin (11). Here we looked for the possible involvement of ErbB3 or ErbB4 in the neurite outgrowth and tyrosine phosphorylation responses we had previously seen associated with overexpression of Neu in PC12 cells. We found that heregulin stimulates the tyrosine phosphorylation of endogenous ErbB3 protein in PC12 cells and that this phosphorylation, like that of Neu, is greatly enhanced in cells that overexpress Neu. Furthermore, we found that overexpressing the ErbB3 protein in PC12 cells led to heregulin-stimulated neurite extension, similar to the phenotypes obtained upon heregulin addition to cells overexpressing the normal Neu protein or when transforming Neu was expressed in PC12 cells. Two obviously important questions were whether the Neu and ErbB3 proteins actually formed a complex in PC12 cells and if this complex formation was stimulated by heregulin. The results of immunoprecipitation experiments using anti-Neu antibody indicated that the addition of heregulin either to PC12 cells overexpressing Neu or to PC12 cells overexpressing ErbB3 led to the co-precipitation of the Neu and ErbB3 proteins. Thus, overall, these results are consistent with a scheme where heregulin-stimulated heterodimer formation between Neu and ErbB3 results in the increased tyrosine phosphorylation of the Neu and ErbB3 proteins (see below) and accounts for the ability of PC12 cells to respond to this growth factor.

Because it has been suggested that one of the primary effector/targets for phosphorylated ErbB3 is the 85-kDa regulatory subunit (p85) of the phosphatidylinositol 3-kinase (18, 19, 20), we set out to determine if heregulin addition to PC12 cells might lead to the formation of an ErbB3-p85 complex. Immunoprecipitation experiments using a specific (precipitating) anti-p85 antibody followed by Western blotting with a specific anti-ErbB3 antibody provided evidence for a direct interaction between these proteins. This interaction was most evident in PC12 cells overexpressing Neu and was heregulin-stimulated. When comparing anti-phosphotyrosine immunoprecipitates with anti-85 immunoprecipitates, where in both cases the resuspended pellets were blotted with the anti-ErbB3 antibody, we found that the ErbB3 band that co-precipitated with p85 was identical in mobility to the slowest mobility ErbB3 band detected in anti-phosphotyrosine precipitates. However, this ErbB3 band was not detected in anti-Neu immunoprecipitates. Thus, although it appears that ErbB3 is phosphorylated at multiple tyrosine residues in a heregulin- and Neu-dependent manner, one of these phosphorylated ErbB3 species forms a stable complex with Neu but not with p85.
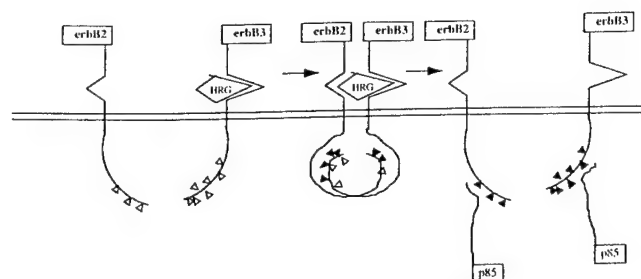

Fig. 7. **A model for heregulin signal transduction involving Neu/ErbB2 and ErbB3 in PC12 cells.** Binding of heregulin (*HRG*) to ErbB3 is followed by formation of a heterodimer of Neu/ErbB2 and ErbB3, within which the Neu tyrosine kinase is activated and ErbB3 becomes phosphorylated. Phosphorylated ErbB3 and Neu/ErbB2 independently complex with p85.

Overall, these results appear to be consistent with the simple scheme depicted in Fig. 7. The binding of heregulin to ErbB3 on the surface of PC12 cells stimulates the formation of a Neu-ErbB3 heterodimer. We would suggest that this results in a higher affinity binding by heregulin (11) and the stimulation of the Neu tyrosine kinase activity. This in turn increases the tyrosine phosphorylation of Neu, probably at multiple sites (*e.g.* Fig. 1), and the trans-phosphorylation of multiple tyrosine residues on ErbB3. The specific mechanism by which heregulin stimulates the phosphorylation of Neu is not clear. One possibility is that Neu is trans-phosphorylated by ErbB3; however, this is problematic because ErbB3 appears to have little or no tyrosine kinase activity (14). It may be that within a Neu-ErbB3 heterodimer, a weak kinase like ErbB3 is still able to phosphorylate Neu because of the immediate proximity of the substrate. Once activated, the Neu tyrosine kinase should be able to trans-phosphorylate ErbB3. The timing of this trans-phosphorylation event apparently is important, because the extent of ErbB3 phosphorylation appears to influence whether or not ErbB3 remains complexed with Neu or forms a new complex with p85. In fact, these findings raise the interesting possibility that a specific, heregulin-stimulated trans-phosphorylation of ErbB3 by Neu leads to the dissociation of ErbB3 from the heregulin-Neu-ErbB3 ternary complex and promotes the specific binding of ErbB3 to a potential target, p85. Such a mechanism would be similar to hormone receptor/G protein-mediated signaling cascades where the G protein first becomes activated within a hormone-receptor-G protein ternary complex, but then the activated G protein dissociates from this complex and seeks out its target/effector molecule.

It also is interesting that a potential signaling cascade involving ErbB3 and p85 within PC12 cells appears to be specifically initiated by heregulin and not by EGF. Although the addition of EGF to PC12 cells overexpressing Neu leads both to an apparent tyrosine phosphorylation of Neu (data not shown) and ErbB3 (Fig. 5A), neither of these phosphorylated proteins appear to form a stable complex with p85. In the future, it will be interesting to see if the heregulin-Neu-ErbB3-p85 pathway contributes to the specificity observed in the cellular responses triggered by heregulin *versus* EGF.

REFERENCES

1. Bargmann, C. I., Hung, M.-C., and Weinberg, R. A. (1986) *Cell* **45**, 649–657
2. Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. (1987) *Science* **235**, 177–182
3. Basler, K., Christen, B., and Hafen, E. (1991) *Cell* **64**, 1069–1082
4. Hill, R. J., and Sternberg, P. W. (1992) *Nature.* **358**, 470–476
5. Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., and Vandlen, R. L. (1992) *Science* **256**, 1205–1211
6. Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Ben Levy, R., and Yarden, Y. (1992) *Cell* **69**, 205–216
7. Marchionni, M. A., Goodearl, A. D. J., Chen, M. S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Koba-

yashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J. B., Hsuan, J. J., Totty, N. F., Otsu, M., McBurney, R. N., Waterfield, M. D., Stroobant, P., and Gwynne, D. (1993) *Nature* **362**, 312–318

8. Falls, D. L., Rosen, K. M., Corfas, G., Lane, W. S., and Fischbach, G. D. (1993) *Cell* **72**, 801–815
9. Gamett, D. C., and Cerione, R. A. (1994) *FEBS Lett.* **351**, 335–339
10. Carraway, K. L., III, Sliwkowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C., and Cerione, R. A. (1994) *J. Biol. Chem.* **269**, 14303–14306
11. Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., III (1994) *J. Biol. Chem.* **269**, 14661–14665
12. Plowman, G. D., Green, J. M., Culouscou, J.-M., Carlton, G. W., Rothwell, V. M., and Buckley, S. (1993) *Nature* **366**, 473–475
13. Tzahar, E., Levkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D., and Yarden, Y. (1994) *J. Biol. Chem.* **269**, 25226–25233
14. Guy, P., Platko, J. V., Cantley, L. C., Cerione, R., and Carraway, K. L. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8132–8136
15. Kim, H. H., Sierke, S. L., and Koland, J. G. (1994) *J. Biol. Chem.* **269**, 24747–24755
16. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) *Cell* **72**, 767–778
17. Carraway, K. L., III, and Cantley, L. C. (1994) *Cell* **78**, 5–8
18. Prigent, S. A., and Gullick, W. J. (1994) *EMBO J.* **13**, 2831–2841
19. Fedi, P., Pierce, J. H., DiFiore, P. P., and Kraus, M. H. (1994) *Mol. Cell. Biol.* **14**, 492–500
20. Soltoff, S. P., Carraway, K. L., III, Prigent, S. A., Gullick, W. J., and Cantley, L. C. (1994) *Mol. Cell. Biol.* **14**, 3550–3558

gene

an international journal focusing on gene cloning and gene structure and function

GENE 09153

Cloning of the rat *ErbB3* cDNA and characterization of the recombinant protein

(HER3; protein tyrosine kinase; Src homology domain; heregulin; oncogene)

Nathan J. Hellyer, Hong-Hee Kim, Charles H. Greaves, Susan L. Sierke and John G. Koland

Department of Pharmacology, University of Iowa College of Medicine, Iowa City, IA 52242-1109, USA

ELSEVIER

Cloning of the rat *ErbB3* cDNA and characterization of the recombinant protein

Nathan J. Hellyer, Hong-Hee Kim, Charles H. Greaves, Susan L. Sierke and John G. Koland

Department of Pharmacology, University of Iowa College of Medicine, Iowa City, IA 52242-1109, USA

SUMMARY

Three cDNA fragments that encoded all but the extreme N terminus of the rat ErbB3 protein were cloned by low-stringency screening of a rat liver cDNA library with a human *ERBB3* probe. The remaining 5′-end of the cDNA was generated by a reverse transcription-polymerase chain reaction method, and a single full-length rat *ErbB3* cDNA was assembled. A comparison of the deduced amino acid (aa) sequences of human and rat ErbB3 was made, and the effects of certain aa substitutions in the putative protein tyrosine kinase domain were considered. The rat *ErbB3* cDNA was subsequently expressed in cultured NIH-3T3 mouse fibroblasts, in which a high level of approx. 180-kDa recombinant ErbB3 (re-ErbB3) was generated. The rat re-ErbB3 produced in transfected fibroblasts was responsive to the polypeptide, heregulin, a known ligand for ErbB3. Challenge of transfected fibroblasts with heregulin stimulated the phosphorylation of rat re-ErbB3 on Tyr residues and promoted its association with the p85 subunit of phosphatidylinositol 3-kinase. Together, these results indicate that a fully functional rat *ErbB3* cDNA has been isolated, and that fibroblast cells expressing this cDNA will be suitable for investigations of the signal transduction mechanism of ErbB3.

INTRODUCTION

The *ERBB* (*HER*) family of human genes has four identified members. The prototypical c-*ERBB* (*HER*)

Correspondence to: Dr. J.G. Koland, Department of Pharmacology, University of Iowa, College of Medicine, Iowa City, IA 52242-1109, USA. Tel. (1-319) 335-6508; Fax (1-319) 335-8930; e-mail: nhellyer@blue.weeg.uiowa.edu

Abbreviations: aa, amino acid(s); Ab, antibody(ies); bp, base pair(s); EGF, epidermal growth factor; ERBB3/ErbB3, human/rat gene (DNA) encoding ErbB3 (also designated *HER3*); GRB2, growth factor receptor-bound protein 2; HRG, heregulin (Neu differentiation factor); oligo, oligodeoxyribonucleotide; PCR, polymerase chain reaction; PI, phosphatidylinositol; PAGE, polyacrylamide gel electrophoresis; PTK, protein tyrosine kinase(s); re-, recombinant; RT, reverse transcription(ase); SDS, sodium dodecyl sulfate; SH, Src homology domain; SHC, Src homology-2 α-collagen related protein; SLIC, single-strand ligation to ss-cDNA; ss, single strand(ed); STP, signal-transducing protein(s); TM, transmembrane; Xaa, any aa.

gene encodes the well studied epidermal growth factor (EGF) receptor and was first identified as the cellular homolog of the v-*ErbB* oncogene of avian erythroblastosis virus (Downward et al., 1984). The EGF receptor consists of a single polypeptide chain that forms an extracellular growth factor binding domain, a short transmembrane (TM) domain, and an intracellular protein tyrosine kinase (PTK) domain. The *ERBB2* (*HER2*) gene, first characterized as a homolog of the rat *Neu* oncogene, also encodes a polypeptide growth factor receptor with PTK activity (Stern et al., 1986). The *ERBB3* (*HER3*) (Kraus et al., 1989; Plowman et al., 1990) and *ERBB4* (*HER4*) (Plowman et al., 1993a) genes have been more recently identified by molecular cloning. The predicted amino acid (aa) sequences of human ErbB3 and ErbB4 are closely related to those of the EGF receptor and ErbB2, which suggests that these two proteins are also receptors with intrinsic PTK activity.

A number of distinct polypeptide ligands that bind and activate ErbB family receptor proteins have now been identified. However, the relationships between the individual ligands and the four ErbB proteins are complex and not fully resolved. Whereas EGF and transforming growth factor α (TGFα) are identified ligands for the EGF receptor (Gill et al., 1987), a variety of polypeptides in the heregulin (HRG) family, which includes the heregulins (Holmes et al., 1992), Neu differentiation factor (NDF) (Wen et al., 1992), and the glial growth factors (Marchionni et al., 1993), have been characterized as ligands for ErbB3. Recent evidence indicates that the high-affinity HRG receptor is actually an ErbB2/ErbB3 heterodimer, with the ErbB3 protein being itself a low-affinity HRG receptor (Sliwkowski et al., 1994; Tzahar et al., 1994). In addition, the ErbB4 protein has been demonstrated to be a high-affinity receptor for HRG (Plowman et al., 1993b; Tzahar et al., 1994).

The intrinsic PTK activity of the ErbB proteins has been generally considered to be essential for their functions in the activation of mitogenic and developmental signal transduction pathways (Schlessinger and Ullrich, 1992). Although the predicted PTK domains of the ErbB proteins have aa sequences that agree well with the consensus sequence for identified PTK (Hanks and Quinn, 1991), human ErbB3 is unique in that three of the aa residues invariantly conserved in PTK are found altered in its predicted PTK domain sequence (Kraus et al., 1989; Plowman et al., 1990). This suggests that ErbB3 might not possess intrinsic PTK activity, and could have a cellular function quite distinct from those of the other ErbB family receptor proteins. To this end, several attempts have been made to demonstrate an intrinsic PTK activity in the *ERBB3* gene product.

Kraus et al. (1993) have documented the constitutive phosphorylation of ErbB3 that is endogenously produced in high levels in certain cultured human breast cancer cell lines. A ligand-dependent activation of the ErbB3 PTK domain in the context of a recombinant chimeric protein that incorporates the extracellular and membrane-spanning domains of the EGF receptor has also been demonstrated (Kraus et al., 1993; Prigent and Gullick, 1994). In contrast, our laboratory (H.-H. K., S.L.S. and J.G.K., data not shown) and others (Guy et al., 1994) have failed to detect intrinsic PTK activity in recombinant and native ErbB3 proteins. To enable the further examination of the catalytic and signal-transducing potentials of ErbB3, we have now isolated a full-length rat *ErbB3* cDNA and generated the corresponding protein in a cultured cell system by gene transfection. In this paper, we present an analysis of this full-length rat *ErbB3* coding sequence and a preliminary characterization of the rat re-ErbB3 protein.

Fig. 1. Deduced aa sequence of rat ErbB3. The rat *ErbB3* cDNA sequence encoded a protein of 1339 aa. Deviations in the human sequence (Kraus et al., 1989; Plowman et al., 1990) are listed below the rat sequence. The putative leader peptide and TM domain are indicated with bold underlines. The symbol (●) indicates three aa conserved in all characterized PTK that are substituted in the human ErbB3 sequence. Other identified sequence elements including several candidate binding sites for STP are indicated by underlining (see Table 1). The cDNA sequence has been deposited in GenBank/EMBL under accession No. U29339. **Methods:** A partial cDNA clone was isolated by screening a rat liver λZAPII library (Stratagene, La Jolla, CA, USA) at reduced stringency with a human *ERBB3* cDNA probe. Overlapping cDNAs were isolated by screening the same library at high stringency with restriction fragment probes derived from successively isolated rat *ErbB3* cDNAs. The remaining 5′-sequence of rat *ErbB3* cDNA was amplified by the 5′-SLIC/RT-PCR method (Dumas et al., 1991). First-strand cDNA synthesis was carried out with rat liver mRNA (1 μg) (Clontech, Palo Alto, CA, USA), Superscript RT (GIBCO-BRL, Grand Island, NY, USA) (10 units/μl), and random hexamer oligo primers (3.3 ng/μl). Four independent PCR products were cloned and sequenced. One product representing the consensus sequence was subcloned with the overlapping cDNA fragments to yield a full-length rat ErbB3 coding sequence.

EXPERIMENTAL AND DISCUSSION

(a) Cloning of the rat *ErbB3* cDNA

Screening of a rat liver bacteriophage λ cDNA library with a human *ERBB3* cDNA probe yielded several cDNA fragments. Sequence analyses indicated that four of these cDNA fragments (*1*) each had high similarity to

the human *ERBB3* cDNA, (*2*) were overlapping, and (*3*) were derived from a single coding sequence, presumably that of rat ErbB3. However, the extreme 5′-end of the rat *ErbB3* message was not represented in these isolated cDNAs. The remaining 5′-end of the rat *ErbB3* cDNA was therefore generated by a RT-PCR method (see Fig. 1).

(b) Deduced aa sequence of rat ErbB3

The 5′-cDNA generated by RT-PCR and three cDNA fragments isolated from the cDNA library were together subcloned to yield a full-length rat *ErbB3* cDNA. The deduced aa sequence of rat ErbB3 and the sequence deviations between human and rat ErbB3 are shown in Fig. 1. Overall there was a 90% aa identity between the human and rat sequences. Two short gaps in the sequence alignment indicated the deletion in the rat sequence of two aa in the presumed TM domain and one aa in the C-terminal autophosphorylation domain. The sequence encoding the putative PTK domain of the rat protein showed a 96% identity with that of the human protein. The rat *ErbB3* gene product is predicted to consist of 1339 aa (147 578 Da).

Comparison of the deduced rat ErbB3 aa sequence with the consensus sequence of the catalytic domains of known PTK (Hanks and Quinn, 1991) showed two substitutions of invariantly conserved aa (see Fig. 1). The residues Cys^{738} and His^{757} of the rat protein, which correspond respectively to Ala^{719} and Glu^{738} of the human epidermal growth factor (EGF) receptor, deviate from the canonical sequence. Notably, Asp^{832} of the rat ErbB3 sequence, which corresponds to Asp^{813} in the EGF receptor sequence, does agree with the consensus sequence, although it is found substituted in the human ErbB3 sequence by Asn^{834}. Given that the mutation of this conserved aspartate residue has been shown to abolish the PTK activity of the EGF receptor (Coker et al., 1994), the lack of this substitution in the rat protein could conceivably alter the catalytic properties of rat ErbB3 relative to those of the human protein. Recently, Prigent and Gullick (1994) employed site-directed mutagenesis to explore the consequences of the substitution in human ErbB3 of His^{759} and Asn^{834} for the normally conserved Glu and Asp residues. The low levels of PTK activity exhibited by wild-type ErbB3 in immune-complex kinase assays were not found to be enhanced by a $His^{759} \rightarrow Glu$ and $Asn^{834} \rightarrow Asp$ double aa substitution.

Other notable structural features are found in the C-terminal domain of rat ErbB3. Of particular interest is the presence of several candidate Tyr residue phosphorylation sites in specific sequence motifs that constitute the recognition sites for SH2 domain (second domain of Src homology) proteins (Cohen et al., 1995; Pawson, 1995). The SH2 domains found in various signal-transducing proteins (STP), such as PI 3-kinase, phospholipase C-γ1, GRB2 and SHC, have been shown to bind to activated growth factor receptors and related proteins that contain phosphorylated Tyr residues. It has become apparent that each distinct SH2 domain protein recognizes phospho-Tyr residues in a specific sequence context (Songyang et al., 1993). The sequence motif Tyr-Xaa-Xaa-Met, the consensus binding site sequence for PI 3-kinase, is found repeated seven times in the C-terminal domain of human ErbB3, and the seven repeats of this sequence are found intact in the C terminus of the deduced rat ErbB3 aa sequence (see Table 1). Two consensus binding sites for the GRB2 protein, Tyr-Met-Asn, are also found in the C-termini of both rat and human ErbB3. Also of interest is the overlapping of PI 3-kinase binding sites with these two GRB2 binding sites in the sequence element Tyr-Glu-Tyr-Met-Asn. Conceivably, these dual-specificity binding sites, depending upon which of the two Tyr residues were phosphorylated, could bind either PI 3-kinase or the GRB2 protein. A potential SH2 domain binding site for Src family PTK, Tyr-Glu-Glu-Met, is also present in both rat and human ErbB3 sequences. Another motif observed in both the rat and human sequences is Tyr-Val-Met-Pro, which if phosphorylated could constitute a binding site for the SH2 domain-containing protein tyrosine phosphatase SH-PTP2. Finally, the binding site for SHC previously identified in human ErbB3 (Prigent and Gullick, 1994) is again found in the rat sequence. The observed cross-species conservation of the aa sequences of these various SH2 domain binding sites is consistent with the assumption that ErbB3 interacts functionally with multiple STP.

Recently it has been determined that the SH3 domains found in various STP bind specifically to certain Pro-rich sequences (Kapeller et al., 1994; Ren et al., 1993). Two such sequences, Pro-Arg-Pro-Pro-Arg-Pro and Pro-Lys-Pro-Pro-Lys-Pro, found in the p85 subunit of PI 3-kinase are part of the identified binding sites for the SH3 domains of Src family PTK (Kapeller et al., 1994). Also, the sequence Pro-Xaa-Xaa-Pro-Pro-Pro-Xaa-Xaa-Pro as found twice in the 3BP-1 protein has been considered a consensus SH3 domain binding site (Ren et al., 1993). The first element, Pro-Arg-Pro-Pro-Arg-Pro, is found within the C terminus of rat ErbB3, with the corresponding sequence in the human protein being Pro-His-Pro-Pro-Arg-Pro (see Table 1). A sequence very similar to the second element, Pro-Leu-His-Pro-Val-Pro-Ile-Met-Pro, is found in the human ErbB3 C terminus, although the corresponding rat sequence, Pro-Leu-His-Pro-Met-Ala-Ile-Val-Pro, is less similar. Presumably these proline-rich sequences of rat ErbB3 could interact directly with SH3 domain proteins, and perhaps in cooperation with the identified SH2 domain binding motifs could mediate high-affinity interactions with proteins

TABLE I

Potential binding sites for SH2 and SH3 domain proteins identified in human and rat ErbB3 aa sequences

SH2/SH3 domain protein[a]	Binding site[b]	Human ErbB3[c]	Rat ErbB3
p85 (PI 3-kinase) (SH2)	Y(M/X)XM	Y^{941}MVM	Y^{939}MVM
		Y^{1054}MPM	Y^{1051}MPM
		Y^{1197}EYMN	Y^{1194}EYMN
		Y^{1222}EYM	Y^{1219}EYM
		Y^{1260}EYMN	Y^{1257}EYMN
		Y^{1276}AAM	Y^{1273}AAM
		Y^{1289}EEM	Y^{1286}EEM
GRB2/Sem-5 (SH2)	YMN	YEY^{1199}MN	YEY^{1196}MN
		YEY^{1262}MN	YEY^{1259}MN
SHC (SH2)	NPXY[d]	$NPDY^{1328e}$	$NPDY^{1325}$
SH-PTP2 (SH2)	YVXP	Y^{1159}VMP	Y^{1156}VMP
Src family kinases (SH2)	YEEM	Y^{1289}EEM	Y^{1286}EEM
p85 (SH3)	P(R/K)PP(R/K)P[f]	P^{1206}HPPRP	P^{1209}RPPRP

[a] Specific proteins containing SH2 or SH3 domains (second or third domain of Src homology, respectively) as indicated in parentheses.

[b] Consensus SH2 domain binding motifs selected from random peptide libraries with specific SH2 domain probes (Songyang et al., 1993). Phosphorylated Tyr residues (Y) in binding site sequences are indicated in bold.

[c] Sites identified in the published aa sequence of human ErbB3 (Kraus et al., 1989; Plowman et al., 1990) with numbering according to Kraus et al. (1989).

[d] Consensus SHC binding site as previously described (Prigent and Gullick, 1994).

[e] Identified SHC binding site in human ErbB3 (Prigent and Gullick, 1994).

[f] From two identified Pro-rich SH3 domain binding site sequences in the p85 subunit of PI 3-kinase (Kapeller et al., 1994).

containing both SH2 and SH3 domains, such as GRB2, p85 and Src family PTK.

(c) Cellular expression of the isolated *ErbB3* cDNA

In order to verify that the full-length cDNA generated by ligation of the several isolated cDNA molecules encoded a protein consistent with the predicted structure of ErbB3, this cDNA was subcloned into a mammalian cell expression vector and transfected into cultured mouse NIH-3T3 fibroblasts. Lysates of the transiently transfected fibroblasts were analyzed by SDS–PAGE and immunoblotting with an ErbB3-specific Ab. A relatively high level of approx. 180-kDa immunoreactive protein was detected in lysates of fibroblasts transfected with the rat *ErbB3* expression vector under conditions of optimal pH (see Fig. 2). Although the molecular mass of recombinant ErbB3 (re-ErbB3) indicated by SDS–PAGE was significantly greater than that predicted by the cDNA sequence, it was similar to that of endogenous ErbB3 of human breast cancer cells (data not shown). Apparently both the rat and human ErbB3 proteins are subject to glycosylation.

(d) Activation of rat re-ErbB3 by the HRG-β peptide

The EGF-related polypeptide HRG-β1, previously characterized as an activating ligand for human ErbB3 (Carraway et al., 1994; Sliwkowski et al., 1994), was tested as an activator of rat re-ErbB3 in transfected NIH-3T3 fibroblasts. While a constitutive phosphorylation of re-ErbB3 was detected by immunoprecipitation with an
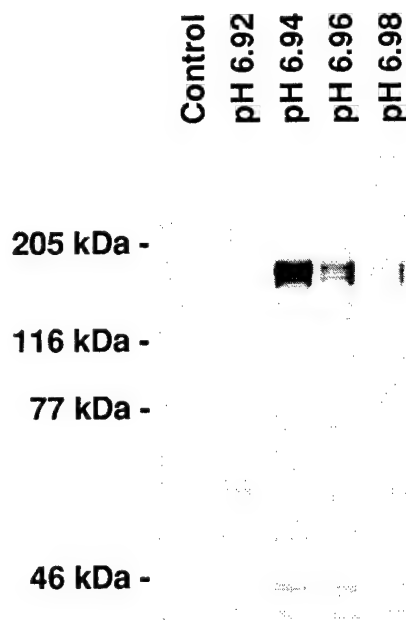
Fig. 2. Transient expression of the rat *ErbB3* cDNA in mouse NIH-3T3 fibroblasts. Subconfluent NIH-3T3 cells in 75-cm^2 flasks were transfected with 20 µg of pcDNA3-*ErbB3* plasmid per flask by a modified Ca·phosphate method (Sambrook et al., 1989). Buffers of slightly varying pH were used to optimize transfection efficiency. At 48 h post-transfection cells were detergent-lysed and samples of the lysates subjected to 0.1% SDS-7% PAGE and immunoblotting with ErbB3-specific monoclonal Ab 2F12 (Kim et al., 1994) (NeoMarkers, Fremont, CA, USA). Immunoblots were visualized with the ECL detection system (Amersham, Arlington Heights, IL, USA). Parental NIH-3T3 cells were analyzed for comparison (Control).

ErbB3-specific Ab and immunoblotting with antiphospho-Tyr, challenge of the transfected fibroblasts with HRG-β1 clearly enhanced the phosphorylation of re-ErbB3 (see Fig. 3). Quantitation of immunoblots by densitometry yielded the average fold-stimulation of re-ErbB3 phosphorylation as 3.3 ± 0.9 $(n = 4)$. Considering that previous studies have demonstrated that ErbB2 and ErbB3 function as coreceptors for the HRG polypeptide (Sliwkowski et al., 1994) and that ErbB4 is also responsive to HRG (Plowman et al., 1993b), it is possible that an endogenous ErbB family member present at low levels in the transfected NIH-3T3 fibroblasts was involved in the observed stimulation of ErbB3 phosphorylation by HRG. As we have in numerous attempts failed to detect intrinsic PTK activity in the rat *ErbB3* gene product (data not shown), we assume that

a distinct PTK(s) was responsible for both the constitutive and HRG-stimulated phosphorylation of re-ErbB3 in the transfected fibroblasts. Notably, we and others have demonstrated an EGF-dependent phosphorylation of ErbB3 in cultured cells containing both the EGF receptor and ErbB3 (Kim et al., 1994; Soltoff et al., 1994).

As previously documented by studies of human ErbB3 (Fedi et al., 1994; Kim et al., 1994; Soltoff et al., 1994), the p85 subunit of PI 3-kinase was found to immunoprecipitate with phosphorylated rat re-ErbB3 (see Fig. 3). Exposure of the transfected fibroblasts to HRG-β1 significantly increased the quantity of p85 detected in ErbB3 immunoprecipitates. The HRG-dependent binding of this STP protein was possibly mediated by the phosphorylation of one or more of the Tyr-Xaa-Xaa-Met p85-binding motifs present in the ErbB3 C terminus.

(e) Conclusions

(*1*) A rat liver cDNA encoding a 1339-aa protein with 90% sequence identity with human ErbB3 has been isolated. Presumably derived from the rat *ErbB3* gene, this cDNA was expressed in cultured mouse NIH-3T3 fibroblasts, in which a high level of the approx. 180-kDa rat re-ErbB3 protein could be generated.

(*2*) The ErbB3 protein isolated by immunoprecipitation of cell lysates was constitutively phosphorylated on Tyr residues. Prior challenge of the cells with HRG-β1 led to an enhanced ErbB3 phosphorylation, which indicated that rat ErbB3 may be a functional HRG receptor.

(*3*) Immunoprecipitation experiments indicated that a fraction of the total p85 protein in the transfected fibroblasts was constitutively associated with rat ErbB3. Challenge of the transfected cells with HRG-β1 augmented the quantity of ErbB3-associated p85. Together these results indicated that the isolated rat *ErbB3* cDNA encodes a functional receptor protein that is responsive to HRG and can transduce signals to the PI 3-kinase pathway.

Fig. 3. Activation of rat re-ErbB3 protein by HRG-β1. Mouse NIH-3T3 fibroblasts were transfected with the pcDNA3-*ErbB3* expression vector (see Fig. 2) and cultured under neomycin (G418) selection. Cells stably expressing the rat *ErbB3* cDNA were serum-starved for 16 h and then incubated for 5 min at 37°C in the presence or absence of an HRG-β1 re-peptide fragment (H.-H. K., J.G.K., and S. Campion, data not shown) incorporating aa 177 to 241 of the HRG-β1 precursor molecule (Holmes et al., 1992) at a concentration of 50 nM. Control cells transfected with the parent pcDNA3 expression vector (Invitrogen, San Diego, CA, USA) were similarly treated. Cell lysates were immunoprecipitated with ErbB3-specific monoclonal Ab 2F12, and immunoprecipitates were subjected to 0.1% SDS-7%-PAGE and immunoblotting with either Ab 2F12 (α-ErbB3), antiphospho-Tyr Ab PY20 (α-P-tyr) (Leinco Technologies, St. Louis, MO, USA), or a polyclonal Ab recognizing the p85 subunit of PI 3-kinase (α-p85) (Transduction Laboratories, Lexington, KY, USA) as indicated.

REFERENCES

Carraway III, K.L., Sliwkowski, M.X., Akita, R., Platko, J.V., Guy, P.M., Nuijens, A., Diamonti, A.J., Vandlen, R.L., Cantley, L.C. and Cerione, R.A.: The *erbB3* gene product is a receptor for heregulin. J. Biol. Chem. 269 (1994) 14303–14306.

284

Cohen, G.B., Ren, R. and Baltimore, D.: Modular binding domains in signal transduction proteins. Cell 80 (1995) 237–248.

Coker, K.J., Staros, J.V. and Guyer, C.A.: A kinase-negative epidermal growth factor receptor that retains the capacity to stimulate DNA synthesis. Proc. Natl. Acad. Sci. USA 91 (1994) 6967–6971.

Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. and Waterfield, M.D.: Close similarity of epidermal growth factor receptor and v-erb-B oncogene protein sequences. Nature (London) 307 (1984) 521–527.

Dumas, J.B., Edwards, M., Delort, J. and Mallet, J.: Oligodeoxyribonucleotide ligation to single-stranded cDNAs: a new tool for cloning 5′ ends of mRNAs and for constructing cDNA libraries by in vitro amplification. Nucleic Acids Res. 19 (1991) 5227–5232.

Fedi, P., Pierce, J.H., Di, F.P. and Kraus, M.H.: Efficient coupling with phosphatidylinositol 3-kinase, but not phospholipase Cγ or GTPase-activating protein, distinguishes ErbB-3 signaling from that of other ErbB/EGFR family members. Mol. Cell. Biol. 14 (1994) 492–500.

Gill, G.N., Bertics, P.J. and Santon, J.B.: Epidermal growth factor and its receptor. Mol. Cell. Endo. 51 (1987) 169–186.

Guy, P.M., Platko, J.V., Cantley, L.C., Cerione, R.A. and Carraway III, K.L.: Insect cell-expressed p180^{erbB3} possesses an impaired tyrosine kinase activity. Proc. Natl. Acad. Sci. USA 91 (1994) 8132–8136.

Hanks, S.K. and Quinn, A.M.: Protein kinase catalytic domain sequence database: identification of conserved features of primary structure and classification of family members. Meth. Enzymol. 200 (1991) 38–62.

Holmes, W.E., Sliwkowski, M.X., Akita, R.W., Henzel, W.J., Lee, J., Park, J.W., Yansura, D., Abadi, N., Raab, H., Lewis, G.D., Shepard, H.M., Kuang, W.J., Wood, W.I., Goeddel, D.V. and Vandlen, R.L.: Identification of heregulin, a specific activator of ^{p185erbB2}. Science 256 (1992) 1205–1210.

Kapeller, R., Prasad, K., Janssen, O., Hou, W., Schaffhausen, B.S., Rudd, C.E. and Cantley, L.C.: Identification of two SH3-binding motifs in the regulatory subunit of phosphatidylinositol 3-kinase. J. Biol. Chem. 269 (1994) 1927–1933.

Kim, H.-H., Sierke, S.L. and Koland, J.G.: Epidermal growth factor-dependent association of phosphatidylinositol 3-kinase with the erbB3 gene product. J. Biol. Chem. 269 (1994) 24747–24755.

Kraus, M.H., Issing, W., Miki, T., Popescu, N.C. and Aaronson, S.A.: Isolation and characterization of ERBB3, a third member of the ERBB/epidermal growth factor receptor family: evidence for overexpression in a subset of human mammary tumors. Proc. Natl. Acad. Sci. USA 86 (1989) 9193–9197.

Kraus, M.H., Fedi, P., Starks, V., Muraro, R. and Aaronson, S.A.: Demonstration of ligand-dependent signaling by the erbB-3 tyrosine kinase and its constitutive activation in human breast tumor cells. Proc. Natl. Acad. Sci. USA 90 (1993) 2900–2904.

Marchionni, M.A., Goodearl, A., Chen, M.S., Bermingham, M.O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J.B., Hsuan, J.J., Totty, N.F., Otsu, M., McBurney, R.N., Waterfield, M.D., Stroobant, P. and Gwynne, D.: Glial growth factors are alter-natively spliced erbB2 ligands expressed in the nervous system. Nature 362 (1993) 312–318.

Pawson, T.: Protein modules and signalling networks. Nature 373 (1995) 573–579.

Plowman, G.D., Whitney, G.S., Neubauer, M.G., Green, J.M., McDonald, V.L., Todaro, G.J. and Shoyab, M.: Molecular cloning and expression of an additional epidermal growth factor receptor-related gene. Proc. Natl. Acad. Sci. USA 87 (1990) 4905–4909.

Plowman, G.D., Culouscou, J.M., Whitney, G.S., Green, J.M., Carlton, G.W., Foy, L., Neubauer, M.G. and Shoyab, M.: Ligand-specific activation of HER4/p180^{erbB4}, a fourth member of the epidermal growth factor receptor family. Proc. Natl. Acad. Sci. USA 90 (1993a) 1746–1750.

Plowman, G.D., Green, J.M., Culouscou, J.M., Carlton, G.W., Rothwell, V.M. and Buckley, S.: Heregulin induces tyrosine phosphorylation of HER4/p180^{erbB4}. Nature 366 (1993b) 473–475.

Prigent, S.A. and Gullick, W.J.: Identification of c-erbB-3 binding sites for phosphatidylinositol 3′-kinase and SHC using an EGF receptor/c-erbB-3 chimera. EMBO J. 13 (1994) 2831–2841.

Ren, R., Mayer, B.J., Cicchetti, P. and Baltimore, D.: Identification of a ten-amino acid proline-rich SH3 binding site. Science 259 (1993) 1157–1161.

Sambrook, J., Fritsch, E.F. and Maniatis, T.: Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Schlessinger, J. and Ullrich, A.: Growth factor signaling by receptor tyrosine kinases. Neuron 9 (1992) 383–391.

Sliwkowski, M.X., Schaefer, G., Akita, R.W., Lofgren, J.A., Fitzpatrick, V.D., Nuijens, A., Fendly, B.M., Cerione, R.A., Vandlen, R.L. and Carraway III K.L.: Coexpression of erbB2 and erbB3 proteins recon-stitutes a high affinity receptor for heregulin. J. Biol. Chem. 269 (1994) 14661–14665.

Soltoff, S.P., Carraway III, K.L., Prigent, S.A., Gullick, W.G., and Cantley, L.C.: ErbB3 is involved in activation of phosphatidylinosi-tol 3-kinase by epidermal growth factor. Mol. Cell. Biol. 14 (1994) 3550–3558.

Songyang, Z., Shoelson, S.E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W.G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R.J., Neel, B. G., Birge, R.B., Fajardo, J.E., Chou, M.M., Hanafusa, H., Schaffhausen, B. and Cantley, L.C.: SH2 domains recognize specific phosphopeptide sequences. Cell 72 (1993) 767–778.

Stern, D.F., Heffernan, P.A. and Weinberg, R.A.: p185, a product of the neu proto-oncogene, is a receptorlike protein associated with tyro-sine kinase activity. Mol. Cell. Biol. 6 (1986) 1729–1740.

Tzahar, E., Levkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D. and Yarden, Y.: ErbB-3 and ErbB-4 function as the respective low and high affinity receptors of all Neu differentiation factor/heregulin isoforms. J. Biol. Chem. 269 (1994) 25226–25233.

Wen, D., Peles, E., Cupples, R., Suggs, S.V., Bacus, S.S., Luo, Y., Trail, G., Hu, S., Silbiger, S.M., Levy, R.B., Koski, R.A., Lu, H.S. and Yarden, Y.: Neu differentiation factor: a transmembrane glycopro-tein containing an EGF domain and an immunoglobulin homology unit. Cell 69 (1992) 559–572.

INSTRUCTIONS TO AUTHORS (Continued)

TITLE PAGE (double spaced; page No. 1) must be prepared in this order: (i) a *brief descriptive title* (lower-case); (ii) about 6–10 *additional key words* (only those **not** already in the title; no obscure abbreviations); (iii) the *name(s) of the author(s)* (lower-case; circle complicated or multiple family names); (iv) *(short) address(es) and Tel. No.(s) of the laboratory where the work was performed* (underline or italicize; specify country); (v) the *corresponding author* (*Correspondence to:* Dr. X.Y. Doe, complete mailing address, Tel. No. and Fax); (vi) single combined footnote with the *present address(es)* and *Tel. No.(s)* of all those author(s) who have moved to a different address; (vii) *abbreviations* (the list of Abbreviations for your paper **must** be included; alphabetically; consult *Gene*'s List of Abbreviations and the pertinent literature, for accepted abbreviations, especially for genes and proteins; list all those used, even if already in *Gene*'s list; create new abbreviations if necessary). Be meticulous with symbols. For proteins, use Roman letters with at least the first letter capitalized (e.g., Hprt, HPRT or βGal); for genes (or DNA) use *italicized* letters [e.g., *lacZ, hprt, HPRT* or *cat* gene (not HPRT or CAT gene)]. Consult the nomenclature (or usage) for your particular organism to use (or create) proper gene/protein abbreviations. Remember: DNA, RNA, cDNA, clones and genes are *expressed*; proteins are *produced* or *synthesized* [e.g., use *cat* expression or CAT production (not CAT expression)]. Mutant (allele) symbols or numbers should not be italicized (e.g., *galK*32 or *gal*-32). Arrange λ genes in the same order as on the map (e.g. λ*b*515*N*7 *c*Its857*S*7). Avoid common mistakes: use μ (not u); *Hind*II (only first three letters italicized); k (not K) for 'kilo' (kDa, kb); Pol*I*k (not Klenow); Ap[R] (superscript, small capital [R]; not Ap').

SUMMARY (on a separate page) should be concise, describing the *purpose* and *results* of the study, and the final specific conclusions. Explain any abbreviations when used for the first time. Use *Gene*'s style for reference in the SUMMARY (i.e., give the complete source reference).

INTRODUCTION must be short (≤1 typed p.) and mainly specify the *background* and *aims* of the work (*not* another SUMMARY).

MATERIALS AND METHODS as a separate section is *not* used in *Gene*, because it is more efficient to list experimental details together with the specific experiments, i.e., in the figure legends and/or tables footnotes, in one of the following manners: (1) add a separate paragraph to the legend and/or footnote entitled **Methods,** and then briefly describe the method(s) used, or (2) simply incorporate all the pertinent experimental details into the legends and/or footnotes (see also TABLES and FIGURES, below). (3) In *rare* cases, a description of a specific novel method (especially in methodological papers) could be incorporated as a special detailed subsection in RESULTS (or EXPERIMENTAL) and DISCUSSION.

EXPERIMENTAL AND DISCUSSION, or RESULTS AND DISCUSSION should point to the data shown in the figures and tables, and briefly discuss their significance. Divide this section into subsections marked, (a), (b), (c), etc., with short subtitles (lower-case, Roman, not underlined). Do not prepare a separate DISCUSSION section; instead, the last subsection subtitled, (x) Conclusions, should be short and divided into points, (*1*), (*2*), (*3*)... [see, e.g., *Gene* 100 (1991) 48–49, or 110 (1992) 5–6].

REFERENCES should be assembled alphabetically and typed double-spaced. They should be referred to by name and year (Harvard System). More than one citation from the same author(s) and year must be identified by the letters, a, b, c, etc., placed after the year of publication. In the text, when referring to a paper by more than two authors, only the name of the first author should be given, followed by a non-italicized 'et al.'. When referring to a personal communication or unpublished paper, all initials and name(s) of all author(s) should be cited, and written permission from those authors should be submitted to your Editor (with a copy to the Editor-in-Chief). Literature references must be in accord with the following examples (also consult recent *Gene* issues):

Westmoreland, B.C., Szybalski, W. and Ris, H.: Mapping of deletions and substitutions in heteroduplex DNA molecules of bacteriophage lambda by electron microscopy. Science 163 (1969) 1343–1348.

Rodriguez, R.L. and Tait, R.C.: Recombinant DNA Techniques: An Introduction, Addison-Wesley, Reading, MA, 1983.

Daniels, D.I., Schroeder, J.L., Szybalski, W., Sanger, F. and Blattner, F.R.: A molecular map of coliphage lambda. In: Hendrix, R.W., Roberts, J.W., Stahl, F.W. and Weisberg, R.A. (Eds.), Lambda II. Cold Spring Harbor Laboratory, Cold Spring Habor, NY, 1983, pp. 469–676.

References must be accurate (rechecked in the library) and follow *Gene*'s style. Journal titles should be abbreviated according to the 'List of Serial Title Word Abbreviations' [1985; ISBN 2-904939-02-8; available from your library or International Serials Data System (ISDS), CIEPS, ISDS International Centre, 20, rue Bachaumont, 75002 Paris (France)]. For reviews with a great number of references, employ the system used in *Gene* Vol. 100 (1991) 13–26.

TABLES should be numbered, I, II, III, etc., and, at the top, bear a short descriptive title. Detailed footnote(s) identified by superscript, [a,b,c,....], usually one for each major column, should be typed double-spaced under the table (for Methods, see FIGURES).

FIGURES (line drawings, including graphs) should be prepared as laser-quality computer printouts. Send *original* printouts, not photos. Only if a laser-quality printer is not available (dot matrix printers are unsatisfactory), should you prepare professionally drawn figures in black ink on white paper. Always use large and bold lettering, and heavy smooth lines, as to permit photographic reduction. Be sure that all symbols in the figure are large and match the explanations in the legend. Sequence figures should be either 60 nucleotides (or amino acids) in width (to fit into a single printed column), or 120–150 nt (or aa) wide (to fit across the entire printed page). Please make figures compact, but still readable. Prepare half-tone Figures (photos of gels, etc.) as collages (=original photos pasted on a laser-printed page). Do NOT re-photograph these collages. In the legends for gel figures, specify gel composition for each panel (e.g., 0.1% SDS-12% PAGE or 8 M urea-6% polyacrylamide or 2.2 M HCOH-1% agarose, etc.). Add bp, nt, kb or kDa symbols above the numerals in marker lanes. The minimum size of figures is 10 × 12 cm and the maximum is 20 × 25 cm. Legends should be typed double-spaced, on separate pages. All pertinent technical details should only be specified in the figure legends and/or table footnotes, and be **detailed** enough **to permit repetition** of the experiments; when appropriate, add a bold subtitle, **Methods.,** to such a description of the experimental details. Please add to legends (for figures) or to footnotes (for tables) all details [temp., time(s), voltage, concentrations, type and % of gel, markers, sizes (bp, kb, kDa), etc.]. **Proofreading** should be done carefully. Please note that it is the Publisher's policy that only printer's errors may be corrected: no changes in, or additions to, the edited manuscript will be accepted.

Page charge. There will be no page charge. Color illustrations are reproduced at the author's expense (the publisher will provide the author with a cost estimate upon submission of the paper).

Reprints. Fifty reprints of each paper will be provided free of charge. Additional copies may be ordered at the prices shown on the Price List which will be sent to the author together with the reprint order form.

Biochemical characterization of the protein tyrosine kinase homology domain of the ErbB3 (HER3) receptor protein

Susan L. SIERKE, Kunrong CHENG, Hong-Hee KIM* and John G. KOLAND†

Department of Pharmacology, University of Iowa College of Medicine, Iowa City, IA 52242, U.S.A.

The putative protein tyrosine kinase domain (TKD) of the ErbB3 (HER3) receptor protein was generated as a histidine-tagged recombinant protein (hisTKD-B3) and characterized enzymologically. CD spectroscopy indicated that the hisTKD-B3 protein assumed a native conformation with a secondary structure similar to that of the epidermal growth factor (EGF) receptor TKD. However, when compared with the EGF receptor-derived protein, hisTKD-B3 exhibited negligible intrinsic protein tyrosine kinase activity. Immune complex kinase assays of full-length ErbB3 proteins also yielded no evidence of catalytic activity. A fluorescence assay previously used to characterize the nucleotide-binding properties of the EGF receptor indicated that the ErbB3 protein was unable to bind nucleotide. The hisTKD-B3 protein was subsequently found to be an excellent substrate for the EGF receptor protein tyrosine kinase, which suggested that *in vivo* phosphorylation of ErbB3 in response to EGF could be attributed to a direct cross-phosphorylation by the EGF receptor protein tyrosine kinase.

INTRODUCTION

Discovered by molecular cloning [1,2], the *ErbB3* gene encodes a member of the ErbB subfamily of receptor protein tyrosine kinases [3]. Like the prototypical epidermal growth factor (EGF) receptor, the ErbB3 protein is predicted to consist of an extracellular ligand-binding domain, a transmembrane domain, an intracellular protein tyrosine kinase domain (TKD) and a C-terminal phosphorylation domain. Despite its structural similarity to other ErbB family receptors (EGF receptor, ErbB2/Neu, ErbB4), the presence of protein tyrosine kinase activity in ErbB3 has been questioned [2], as the deduced amino acid sequence of the protein shows three substitutions for residues invariantly conserved in all protein tyrosine kinases with known sequence [4]. Efforts to resolve this question have led to conflicting results. Two groups have detected ligand-stimulated protein tyrosine kinase activity in a chimaeric EGF receptor/ErbB3 protein, and concluded that the ErbB3 cytosolic domain possesses intrinsic catalytic activity [5,6]. However, a third group found negligible protein kinase activity in a recombinant bovine ErbB3 protein [7].

Recently, the ErbB3 protein has been shown to bind EGF-related polypeptides in the neuregulin (heregulin) family [8–10]. In cultured cells expressing ErbB3, the protein has been seen to be phosphorylated on tyrosine residues in response to EGF or neuregulin [11–13]. As this phosphorylation is dependent on the co-expression of either the EGF receptor or ErbB2 [14–21], it has been considered that the ErbB3 protein may be a physiological substrate for the protein tyrosine kinase activities of the EGF receptor and ErbB2. Indeed it appears that the ErbB3 protein may form receptor heterodimers with either the EGF receptor or ErbB2 protein (reviewed in [22–24]). The role of any intrinsic protein tyrosine kinase activity of ErbB3 in the phosphorylation of ErbB3 and its associated ErbB family members within the context of receptor heterodimers remains unclear.

In order to assess the catalytic potential of ErbB3, the cytosolic domain of the protein and that of the well-characterized EGF receptor were generated by use of the baculovirus/insect cell expression system. The purified recombinant proteins were characterized by CD spectroscopy, protein tyrosine kinase activity assays and a recently described nucleotide-binding assay [25]. The recombinant ErbB3 protein was seen to be devoid of intrinsic protein tyrosine kinase activity, and indeed appeared unable to bind nucleotide. The ErbB3 cytosolic domain was subsequently found to be an excellent substrate for the EGF receptor protein tyrosine kinase. Together these results indicated that the observed phosphorylation of ErbB3 in the cellular context might be effected by the protein tyrosine kinase activities of other ErbB family members.

EXPERIMENTAL

Cell lines and reagents

All cell lines were purchased from American Type Culture Collection and cultured as recommended. 2′(3′)-O-(2,4,6-Trinitrophenyl)adenosine 5′-triphosphate (TNP-ATP) was obtained from Molecular Probes. [γ-^{32}P]ATP ($\sim$ 3000 Ci/mmol) was supplied by Dupont–New England Nuclear. ErbB3-specific (2F12) and EGF-receptor-specific (LA1) monoclonal antibodies were purchased from NeoMarkers and Upstate Biotechnology respectively. Phosphotyrosine-specific monoclonal antibody (PY20) was obtained from Leinco Technologies. Horseradish

peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents were purchased from Amersham. The fusion protein GST–TK7 [26] and NIH-3T3 cells expressing the rat ErbB3 cDNA [27] have been previously described.

Expression of EGF receptor and ErbB3 TKD forms

Recombinant human EGF receptor and rat ErbB3 TKD forms, each with a Met-His-His-His-His-His-His leader peptide, were expressed with the baculovirus/insect cell system. The hisTKD38-coding sequence was generated from the EGF receptor cDNA in pMMTV-ER [28] by PCR with the primers 5′-TGCTCTAGACCATGCACCACCACCACCACCACCGA-AGGCGCCACATCGTTCGG-3′ (forward) and 5′-CCCCCG-GGCTAGTTGGAGTCTGTAGGACTTGGCAA-3′ (reverse). The forward primer included an XbaI restriction site, a start codon (underlined) and six His codons, as well as the coding sequence for amino acid residues 645–651 of the EGF receptor. The reverse primer was complementary to the coding sequence for residues 965–972 of the EGF receptor, and introduced a stop codon (underlined) and a SmaI restriction site into the PCR product. The resulting PCR product was subcloned into the baculovirus transfer vector pAcYMP1 [29] to yield pAc-TKD38. A baculovirus transfer vector for hisTKD61 (pAc-TKD61) was generated by cloning a cDNA fragment encoding the EGF receptor C-terminus into pAc-TKD38.

The coding sequence for the rat ErbB3 TKD was amplified by PCR from a previously characterized rat ErbB3 cDNA clone, pBS-rB3 [27]. The forward primer, 5′-TGCTCTAGACCATG-CACCACCACCACCACCACCGAATTCGGATTCAGAA-CAAAAGGGCTA-3′, included an XbaI site, a start codon (underlined), six His codons and the codons for amino acid residues 668–674 of ErbB3. The reverse primer, 5′-ACAA-GCTGCAGAGATGAC-3′, was complementary to a coding sequence within the rat ErbB3 cDNA downstream of a unique NdeI restriction site. The resulting PCR product was cloned into pBS-rB3 to yield a cDNA encoding the hisTKD-B3 protein, which was then subcloned into pAcYMP1. The authenticity of the PCR-amplified sequences present in each transfer vector was directly verified by DNA sequencing.

The purified baculovirus transfer vectors were co-transfected with BaculoGold baculovirus DNA (Pharmingen) into cultured Sf21 cells [30]. Recombinant baculovirus clones were isolated by an end point dilution method [31], and viral clones expressing high levels of the recombinant TKDs were identified by immuno-blotting lysates of virally infected Sf21 cells.

For large-scale preparation of recombinant proteins, Sf21 cells were grown in spinner flask culture (125 ml) to a density of $(1–2) \times 10^6$ cells/ml, then infected with recombinant virus (~ 10 plaque-forming units/cell) [30]. At 48 h after infection, cells were harvested and washed gently in 20 ml of insect cell lysis buffer (20 mM Tris/HCl, 0.5 M NaCl, 5 mM imidazole, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM PMSF, pH 7.9) supplemented with 250 mM sucrose. The cells were resuspended in 10 ml of sucrose-free lysis buffer and sonicated. The homogenate was clarified by centrifugation for 20 min at 80000 g, and supplemented with Triton X-100 to a final concentration of 0.05%. The solution was applied to a 5 ml iminodiacetic acid–Sepharose 6B column (Sigma) that had been charged with 50 mM nickel sulphate and equilibrated with binding buffer (20 mM Tris/HCl, 0.5 M NaCl, 0.05% Triton X-100, pH 7.9) supplemented with 5 mM imidazole. The column was washed with ten column volumes of binding buffer (5 mM imidazole) and six column volumes of binding buffer supplemented with 60 mM imidazole, then eluted with binding buffer

supplemented with 250 mM imidazole. Peak fractions in the eluate were identified by protein assays [32] and pooled. Free imidazole was removed by extensive dialysis against TKD dialysis buffer [20 mM Tris/HCl, 100 mM NaCl, 10% (v/v) glycerol, 0.05% Triton X-100, pH 7.9]. The purified TKD forms ($\sim 95\%$ pure, typically 1–2 mg of total protein) were supplemented with dithiothreitol to 1 mM and glycerol to 45% (v/v) and stored at -20 °C. All purification steps were carried out at 4 °C or on ice. In protein purifications for CD measurements, Triton X-100 was omitted from the column elution and final dialysis buffers.

In vitro protein tyrosine kinase assays

TKD proteins (0.25 μM) were incubated for 5 min at room temperature in TKD dialysis buffer (36 μl total volume) with 15 μM [γ-^{32}P]ATP ($\sim 10^4$ c.p.m./pmol), 10 mM MnCl$_2$ or MgCl$_2$, and 0.1% Triton X-100 added to the indicated concentrations. After quenching of the reactions by the addition of SDS/PAGE sample buffer, phosphoproteins were resolved by SDS/PAGE [33] and detected by autoradiography. Assays of exogenous peptide-phosphorylation activity included GST–TK7 (5 μg), a glutathione S-transferase fusion protein incorporating residues 943–1011 of the EGF receptor protein that has previously been shown to be an excellent protein tyrosine kinase substrate [26].

The cross-phosphorylation of the ErbB3 TKD (hisTKD-B3) by the truncated EGF receptor TKD (hisTKD38) was assayed as described above, except that the incubations were carried out for 15 min at room temperature. Phosphoproteins were then identified either by immunoblotting with the phosphotyrosine-specific antibody PY20 or by autoradiography. The kinetics of the cross-phosphorylation reaction were assayed by incubating hisTKD38 (0.25 μM) in the presence of 10 mM MnCl$_2$, 15 μM [γ-^{32}P]ATP and various concentrations (0–2.5 μM) of hisTKD-B3 for 5 min at room temperature in TKD dialysis buffer. The final glycerol concentrations of the samples were adjusted to a constant 30% (v/v). The ^{32}P-labelled phosphoproteins were resolved by SDS/PAGE, identified by autoradiography, and quantified by scintillation counting of bands excised from dried gels. V_{max} and K_m were determined by the fitting of rate equations with a non-linear least-squares minimization alogrithm [34], and the hyperbolic curve generated is shown in Figure 6(B).

In immune complex kinase assays, immunoprecipitates were incubated with [γ-^{32}P]ATP ($\sim 10^4$ c.p.m./pmol) for 10 min at room temperature. Reactions contained 40 mM Hepes/Na (pH 7.4), 0.05% Triton X-100, 10 mM MnCl$_2$, or 10 mM MgCl$_2$ and 3 mM MnCl$_2$, and 17 μM ATP. Then $5 \times$ SDS/PAGE sample buffer was added to stop the reactions, and the samples were subjected to electrophoresis and autoradiography.

Fluorescence spectroscopic analysis of nucleotide binding

Binding of the TNP-ATP nucleotide analogue to the recombinant TKDs was analysed by a recently described fluorescence assay [25]. Briefly, fixed concentrations of recombinant protein were titrated with increasing concentrations of TNP–ATP (0–7.5 μM) as the fluorescence of the nucleotide was recorded. Fluorescence titration data were corrected for the contribution of both free and non-specifically bound TNP-ATP, as determined by titrations performed with the inclusion of excess ATP, and for inner filter quenching effects observed at high TNP-ATP concentrations. Dissociation constants for TNP-ATP binding were subsequently determined by fitting of a theoretical binding equation to the titration data [25].

CD spectroscopic measurements

UV CD spectra of recombinant proteins were recorded with an Aviv 62DS instrument with solutions of 2 μM protein in 10 mM Tris/HCl/50 mM NaCl/25 % (v/v) glycerol, pH 7.9, held in 2 mm cells thermostatically controlled at 4 °C. A solvent blank spectrum was subtracted from each protein spectrum. Analysis of CD spectra for determination of the content of secondary-structural elements was carried out with the aid of spectral decomposition software [35].

RESULTS

Generation and characterization of recombinant EGF receptor and ErbB3 TKDs

In order to compare the catalytic properties of the EGF receptor and the ErbB3 protein, the TKDs of these receptors were expressed as recombinant proteins with the baculovirus/insect cell system. Baculovirus expression vectors for two distinct EGF receptor TKD forms, one with an authentic C-terminus (hisTKD61) and one with a highly truncated C-terminus (hisTKD38), and a full-length ErbB3 TKD form (hisTKD-B3) were constructed (Figure 1). The three recombinant TKDs were expressed in Sf21 cells, and each of these proteins was effectively purified by Ni^{2+}-chelating column chromatography (Figure 2A).

The secondary structures of the purified recombinant proteins were analysed by CD spectroscopy. The spectra of the C-terminally complete hisTKD61 and hisTKD-B3 proteins were qualitatively similar, and spectral decomposition analysis [35]
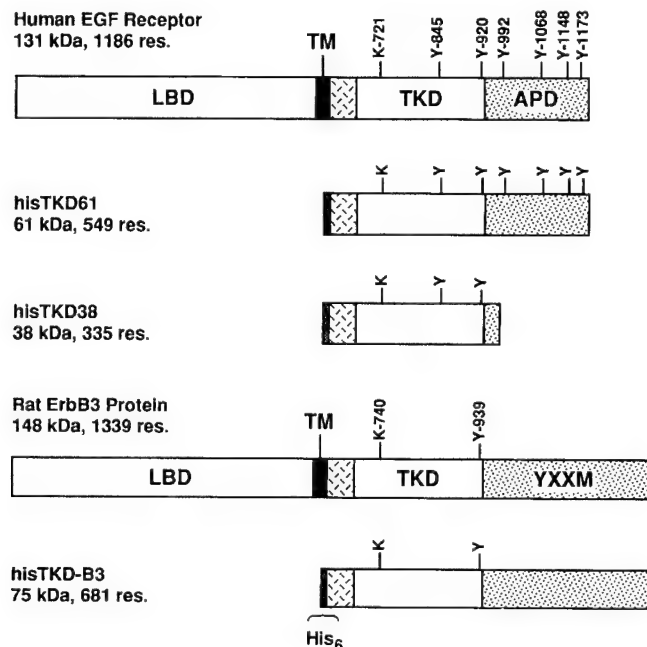
Figure 1   Schematic structures of recombinant EGF and ErbB3 receptor TKDs

The putative functional domains of the two receptor proteins are indicated: ligand-binding domain (LBD), transmembrane domain (TM), protein tyrosine kinase domain (TKD) and autophosphorylation domain (APD). Candidate phosphorylation sites in the EGF receptor are identified, and YXXM labels the C-terminal domain of ErbB3 that contains seven repetitions of the consensus phosphatidylinositol 3-kinase-binding site, Tyr-Xaa-Xaa-Met. The Met-His-His-His-His-His-His leader peptide introduced into each of the recombinant proteins is also indicated (His_6).
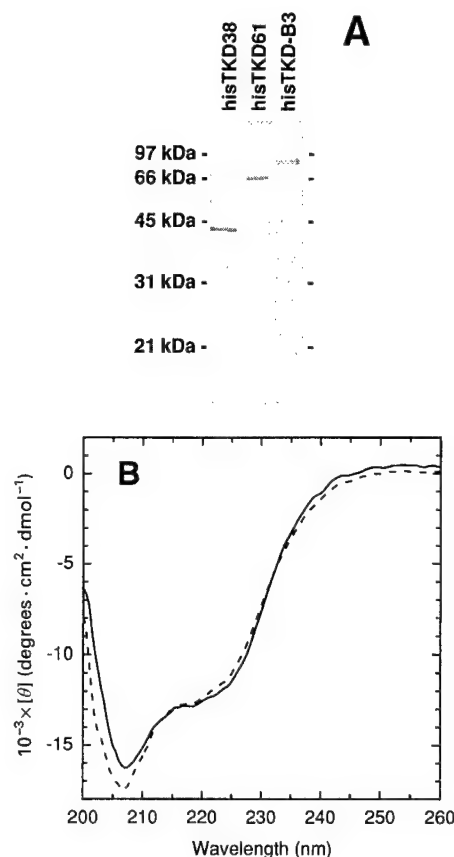
Figure 2   Characterization of EGF receptor and ErbB3 TKDs by SDS/PAGE and CD spectroscopy

(**A**) Recombinant TKD proteins generated with the baculovirus/insect cell system were purified to near-homogeneity by Ni^{2+}-chelating column chromatography (see the Experimental section). SDS/PAGE analysis with silver staining of 0.5 μg samples of the three TKD forms is shown. (**B**) CD spectra of EGF receptor (——) and ErbB3 (————) TKDs were recorded and analysed for context of secondary-structural elements (see the Experimental section). Percentages of α-helix, β-sheet, β-turn and random elements were 41, 35, 10 and 14% respectively for the hisTKD61 protein, and 40, 38, 9 and 13% respectively for the hisTKD-B3 protein.

indicated similar contents of α-helix, β-sheet, β-turn and random structural elements (Figure 2B). Given that the EGF receptor-derived hisTKD61 protein was found to possess a catalytic activity comparable with that of the native EGF receptor protein (results not shown), it was assumed that this recombinant protein was folded in a native conformation. The similarity of the CD spectrum of the hisTKD-B3 protein to that of the hisTKD61 protein then suggested that the ErbB3-derived protein also assumed a native conformation.

Catalytic activities of recombinant EGF receptor and ErbB3 TKDs

Previous studies of a full-length EGF receptor TKD expressed in the baculovirus/insect cell system indicated that the hisTKD61 protein would be an active protein tyrosine kinase showing selectivity for Mn^{2+} over Mg^{2+} as an activating metal ion [36,37]. The hisTKD38 protein was also expected to be fully active, although it was expected that this truncated protein would lack the strong autophosphorylation activity of the full-length TKD. The autophosphorylation and substrate phosphorylation activities of the two recombinant EGF receptor TKD forms were compared with those of the ErbB3-derived protein (Figure 3).
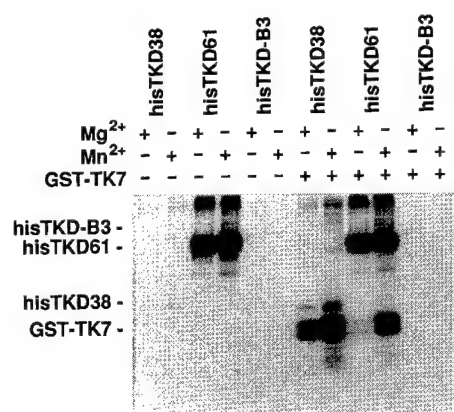
Figure 3 Autophosphorylation and substrate-phosphorylation activities of the EGF receptor and ErbB3 TKDs

Each of the TKD proteins (0.25 μM) was incubated for 5 min at room temperature in the presence of 15 μM [γ-^{32}P]ATP, and either 10 mM MgCl$_2$ or 10 mM MnCl$_2$ as indicated. TKD autophosphorylation was analysed by SDS/PAGE and autoradiography. Substrate phosphorylation activities of the TKD proteins were similarly assayed with the inclusion of 5 μg of the protein substrate GST–TK7 in the incubation as indicated. The GST–TK7 protein shows multiple bands when phosphorylated.

These experiments employed a recombinant fusion protein (GST–TK7) known to be a substrate for the EGF receptor and c-Src protein tyrosine kinases [26], and both Mg^{2+} and Mn^{2+} were tested as activators of the phosphorylation reactions.

Whereas the hisTKD61 protein showed a strong autophosphorylation, autophosphorylation of the hisTKD38 and hisTKD-B3 proteins was much weaker. Phosphoamino acid analyses (results not shown) indicated that, whereas the weak autophosphorylation of the hisTKD38 protein corresponded to the incorporation of phosphotyrosine, the hisTKD-B3 protein was not phosphorylated on tyrosine residues (see also Figure 6A). Each of the three TKD forms was phosphorylated to a very small extent on serine and threonine, which was apparently due to a contaminating serine/threonine kinase activity. Significantly, the substrate-phosphorylation activity of the hisTKD-B3 protein was negligible compared with that of the EGF receptor-derived TKDs. Several other attempts to detect protein tyrosine kinase activity in the ErbB3 TKD also yielded negative results. For example, when a distinct ErbB3 TKD lacking the hexa-His leader peptide was generated with a vaccinia virus expression system, intrinsic protein tyrosine kinase activity was again not evident (results not shown).

Catalytic activity of the full-length ErbB3 protein *in vitro*

The protein tyrosine kinase activity of the full-length ErbB3 protein was also assessed. Here, the native ErbB3 protein was immunoprecipitated from cells expressing the protein at a high level either as a consequence of gene transfection (3T3-B3 cells) or tumorigenesis (MDA-MB-453 and SK-BR-3) (Figure 4A). For comparison, the EGF receptor was immunoprecipitated from MDA-MB-468 cells. Immunoprecipitated proteins were incubated with [γ-^{32}P]ATP and bivalent metal ions. As expected, EGF receptor immune complexes showed strong autophosphorylation. In contrast, ErbB3 immunoprecipitates exhibited negligible autophosphorylation activity (Figure 4B). Neither varying the assay conditions nor stimulating with the ligand neuregulin led to the detection of ErbB3 kinase activity (results not shown). Both rat and human ErbB3 proteins were
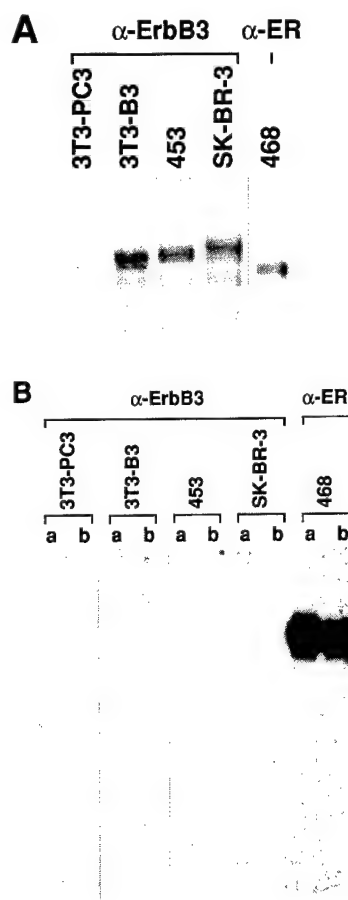
Figure 4 Autophosphorylation activities of full-length EGF receptor and ErbB3 proteins

(A) Immunoprecipitation of ErbB3 and EGF receptor from cultured human breast cancer cell lines and NIH-3T3 cells expressing recombinant rat ErbB3 protein. Detergent lysates were prepared from NIH-3T3 fibroblasts transfected with the parent pcDNA3 expression vector (3T3-PC3) or the pcDNA3-ErbB3 vector (3T3-B3) and three human breast cancer cell lines: MDA-MB-453 (453), MDA-MB-468 (468) and SK-BR-3. Aliquots of each lysate (4 mg of protein) were precleared with Protein G–agarose, then immunoprecipitated with either ErbB3-specific antibody 2F12 (α-ErbB3) or EGF-receptor-specific antibody LA1 (α-ER) as indicated. After two washes, precipitates were resuspended and one-half of each sample was analysed by SDS/PAGE and immunoblotting with the immunoprecipitating antibodies. (B) Immune complex kinase assays performed with ErbB3 and EGF receptor immunoprecipitates. One-fifth of each suspended immunoprecipitate analysed in (A) was incubated for 10 min at room temperature in the presence of 17 μM [γ-^{32}P]ATP and either 10 mM MnCl$_2$ (lanes a) or a mixture of 10 mM MgCl$_2$ and 3 mM MnCl$_2$ (lanes b). The phosphoproteins were resolved by SDS/PAGE and identified by autoradiography.

tested here, as the transfected NIH-3T3 cells expressed the rat ErbB3 protein and the cancer cell lines used were derived from human breast carcinomas.

Nucleotide-binding properties of EGF receptor and ErbB3 TKD proteins

Previously, we have shown that the fluorescent nucleotide analogue TNP–ATP binds to recombinant EGF receptor TKD forms, and that this binding can be conveniently monitored by measuring the enhancement of TNP-ATP fluorescence that occurs on binding to the TKD [25]. The Mn·TNP-ATP complex was found to be a functional substrate for the EGF receptor protein tyrosine kinase, which apparently mimics the authentic substrate Mn·ATP. The TNP-ATP nucleotide binding exhibited
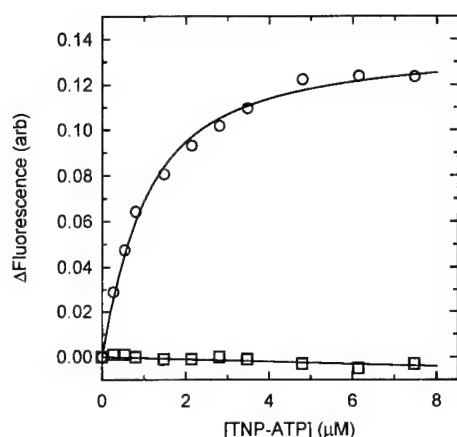
Figure 5   Nucleotide-binding properties of recombinant EGF receptor and ErbB3 TKDs

The interaction of the nucleotide analogue TNP-ATP with the recombinant TKD proteins was analysed by fluorescence spectroscopy as previously described [25]. Whereas the EGF-receptor-derived hisTKD61 protein (○) showed a high-affinity interaction with TNP-ATP ($K_d = 0.75 \pm 0.24$ μM), the ErbB3-derived hisTKD-B3 protein (□) showed no interaction.

by the EGF-receptor-derived hisTKD61 protein was directly compared with that of the hisTKD-B3 protein (see Figure 5). Whereas the hisTKD61 protein bound the nucleotide analogue with a dissociation constant in the micromolar range ($K_d = 0.75 \pm 0.24$ μM), there was no detectable interaction of the nucleotide analogue with the ErbB3-derived protein. Failure of the fluorescent nucleotide analogue to interact with hisTKD-B3 precluded attempts to address directly the ATP and Mn·ATP binding properties of this protein. However, the inability of the ErbB3 protein to bind the nucleotide analogue was certainly consistent with its observed lack of protein tyrosine kinase activity. In related studies (results not shown), a truncated ErbB3 TKD protein lacking the C-terminal phosphorylation domain was found to associate with TNP-ATP and ATP, but did not detectably interact with Mn·TNP-ATP and also showed no catalytic activity.

ErbB3 as a protein tyrosine kinase substrate

In our earlier work [38], C-terminal sequences of the ErbB3 receptor protein were found to be excellent substrates for the EGF receptor protein tyrosine kinase with K_m values ranging from 1 to 30 μM. This suggested that if the ErbB3 receptor was not itself an active protein kinase, it might serve as a substrate for another receptor protein kinase in the ErbB family. To examine the potential for EGF receptor/ErbB3 cross-phosphorylation, the EGF receptor-derived hisTKD38 protein was incubated with the ErbB3-derived hisTKD-B3 under phosphorylating conditions (Figure 6A). Whereas the hisTKD38 and hisTKD-B3 proteins alone showed negligible autophosphorylation activities when compared with the C-terminally complete hisTKD61 protein, hisTKD-B3 was strongly phosphorylated on incubation with hisTKD38. This phosphorylation could be detected by either autoradiography of ^{32}P-labelled proteins or immunoblotting with anti-phosphotyrosine (Figure 6A). The K_m and V_{max} for phosphorylation of the hisTKD-B3 substrate by the hisTKD38 protein kinase were approx. 0.5 μM and 1.4 nmol/min per mg respectively (Figure 6B). Hence the hisTKD-B3 protein exhibited a K_m value among the lowest documented for substrates for the EGF receptor protein tyrosine kinase. The hisTKD-B3
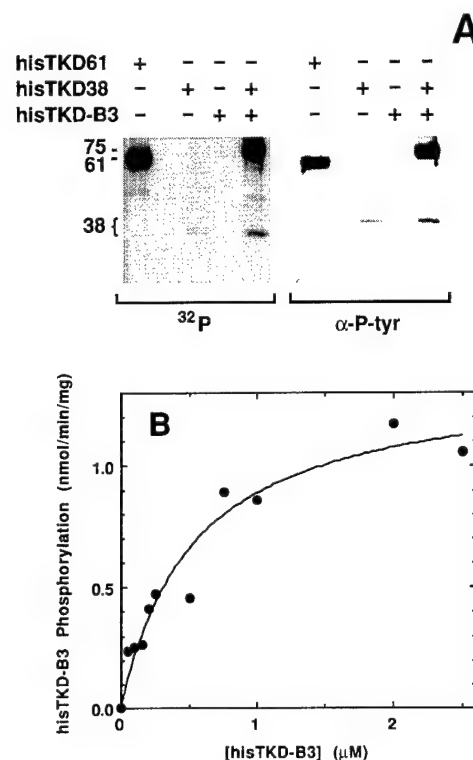
Figure 6   Autophosphorylation and cross-phosphorylation activities of EGF receptor and ErbB3 TKDs

(A) The C-terminally complete EGF receptor TKD (hisTKD61), the truncated EGF receptor TKD (hisTKD38) and the ErbB3 TKD (hisTKD-B3) (see Figure 1) (each at 0.25 μM concentration) were incubated either separately or together as indicated for 15 min at room temperature in the presence of 10 mM $MnCl_2$ and either 15 μM [γ-^{32}P]ATP (left panel) or 15 μM ATP (right panel). Phosphorylation was analysed by SDS/PAGE and autoradiography (left panel) or by anti-phosphotyrosine immunoblotting (right panel). (B) The kinetics of phosphorylation of hisTKD-B3 by hisTKD38 were analysed by incubating hisTKD38 (0.25 μM) in the presence of 0–2.5 μM hisTKD-B3 for 5 min at room temperature as in (A). ErbB3 phosphorylation was assayed by SDS/PAGE, autoradiography and scintillation counting of excised gel bands. The hyperbolic curve best fitting the experimental data is shown ($K_m = 0.54$ μM; $V_{max} = 1.4$ nmol/min per mg).

protein was also efficiently phosphorylated by the C-terminally complete EGF receptor protein kinase, hisTKD61 (results not shown), although the similar SDS/PAGE mobilities of the hisTKD-B3 and hisTKD61 proteins precluded a quantitative analysis of this phosphorylation reaction.

DISCUSSION

The *ErbB3* gene product has been predicted to be a receptor protein tyrosine kinase similar in structure and function to other EGF receptor family members [1,2]. We have attempted to detect intrinsic protein tyrosine kinase activity in the ErbB3 protein by various approaches. As the protein tyrosine kinase domains of a variety of other receptors have been produced in catalytically active form with the baculovirus system [29,39,40–42], we used this sytem in the generation of an ErbB3 cytosolic domain protein (hisTKD-B3). CD spectroscopic measurements indicated that the hisTKD-B3 protein was folded in a conformation similar to that of the corresponding EGF receptor cytosolic domain (hisTKD61), which displayed robust catalytic activity. However, the recombinant ErbB3 protein exhibited negligible catalytic activity under the same experimental conditions (Figure

3). Immune complex kinase assays of full-length ErbB3 proteins also failed to demonstrate intrinsic kinase activity (Figure 4). These results led to the conclusion that the ErbB3 protein is not intrinsically a protein kinase.

The potential of the ErbB3 protein to bind nucleotide substrates was assessed with the aid of the fluorescent nucleotide analogue TNP-ATP, which has previously been used to characterize the nucleotide-binding properties of the EGF receptor TKD [25]. Whereas the EGF receptor-derived hisTKD61 protein again bound TNP-ATP with high affinity (Figure 5), there was no observed enhancement of TNP-ATP fluorescence in the presence of the hisTKD-B3 protein. Because there was no apparent interaction of the analogue with hisTKD-B3, it was not possible to use this assay to investigate the potential interaction of ErbB3 with the authentic substrate Mn·ATP. In a previous study of recombinant bovine ErbB3 [7], the receptor protein was seen to be specifically labelled by 5′-p-fluorosulphonylbenzoyladenosine, although again no evidence for intrinsic kinase activity was obtained. Given that the ErbB3 cytosolic domain here did not interact with the analogue TNP-ATP and also showed no catalytic activity, it is reasonable to suspect that the ErbB3 protein may be unable to bind ATP in the same manner as other protein tyrosine kinases.

The apparent absence of catalytic activity and failure to bind nucleotide substrate might be explained by the occurrence of non-conservative amino acid substitutions in the putative protein tyrosine kinase domain of ErbB3. Specifically, the residues Cys-721, His-740 and Asn-815 in human ErbB3 [2] correspond to Ala, Glu and Asp respectively in all other known protein tyrosine kinases [4]. Sequencing of the rat ErbB3 cDNA has revealed an Asp residue corresponding to Asn-815 in human ErbB3 [27], which suggested that the rat ErbB3 protein, unlike human ErbB3, might possess kinase activity. However, neither rat nor human ErbB3 showed evidence of catalytic activity in this study (Figure 4).

A previous study of the bovine ErbB3 protein also yielded no indication of significant kinase activity [7]. However, an apparent intrinsic protein tyrosine kinase activity was detected in other investigations of the human ErbB3 protein [5,6]. In these latter studies, *in vitro* phosphorylation of ErbB3 in an immune complex [5] and EGF-stimulated *in vivo* phosphorylation of a chimaeric receptor consisting of the extracellular domain of EGF receptor and cytosolic domain of ErbB3 were demonstrated [5,6]. It is possible that this observed ErbB3 phosphorylation resulted from the action of an associated non-ErbB3 kinase. For example, the *in vivo* and *in vitro* phosphorylations of kinase-deficient mutant forms of the EGF receptor have been demonstrated [43,44], and an ectopically expressed kinase-deficient EGF receptor mutant was shown to be cross-phosphorylated by endogenous wild-type EGF receptors [45].

The ErbB3 protein has been shown to function with ErbB2/neu as a high-affinity coreceptor for the neuregulin (heregulin) peptides [8,10]. Also, the EGF-dependent phosphorylation of the ErbB3 protein in human cancer cells expressing high levels of both EGF receptor and ErbB3 has been documented [11,12]. A variety of recent evidence is consistent with a general model in which pairs of distinct ErbB family receptor proteins function as receptor heterodimers [23]. In this model, receptor heterodimerization provides a mechanism for diversifying the signal-transduction pathways activated by polypeptide growth factors in the EGF family. As specific phosphorylated tyrosine residues within the unique C-termini of the ErbB family members have been shown to function as docking sites for distinct signal-transducing proteins such as phospholipase C, phosphatidyl-inositol 3-kinase, Grb2 and Shc [46], receptor phosphorylation in the context of heterodimers is a critical event in ErbB family receptor signal transduction. If devoid of intrinsic protein tyrosine kinase activity, the ErBB3 protein would be phosphorylated only in association with other ErbB family receptor proteins. Our observation that the ErbB3 protein was an excellent substrate for the EGF receptor *in vitro* (Figure 6) is consistent with the assumption that the documented *in vivo* phosphorylation of ErbB3 in response to either EGF or neuregulin results directly from the action of other ErbB family protein tyrosine kinases.

REFERENCES

1 Kraus, M. H., Issing, W., Miki, T., Popescu, N. C. and Aaronson, S. A. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 9193–9197
2 Plowman, G. D., Whitney, G. S., Neubauer, M. G., Green, J. M., McDonald, V. L., Todaro, G. J. and Shoyab, M. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 4905–4909
3 Fantl, W. J., Johnson, D. E. and Williams, L. T. (1993) Annu. Rev. Biochem. **62**, 453–481
4 Hanks, S. K. and Quinn, A. M. (1991) Methods Enzymol. **200**, 38–62
5 Kraus, M. H., Fedi, P., Starks, V., Muraro, R. and Aaronson, S. A. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 2900–2904
6 Prigent, S. A. and Gullick, W. J. (1994) EMBO J. **13**, 2831–2841
7 Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A. and Carraway, III, K. L. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 8132–8136
8 Carraway, III, K. L., Sliwkowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C. and Cerione, R. A. (1994) J. Biol. Chem. **269**, 14303–14306
9 Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L. and Carraway, III, K. L. (1994) J. Biol. Chem. **269**, 14661–14665
10 Tzahar, E., Levkowitz, G., Karaunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D. and Yarden, Y. (1994) J. Biol. Chem. **269**, 25226–25233
11 Soltoff, S. P., Carraway, III, K. L., Prigent, S. A., Gullick, W. G. and Cantley, L. C. (1994) Mol. Cell. Biol. **14**, 3550–3558
12 Kim, H.-H., Sierke, S. L. and Koland, J. G. (1994) J. Biol. Chem. **269**, 24747–24755
13 Kita, Y. A., Barff, J., Luo, Y., Wen, D., Brankow, D., Hu, S., Liu, N., Prigent, S. A., Gullick, W. J. and Nicolson, M. (1994) FEBS Lett. **349**, 139–143
14 Peles, E., Ben-Levy, R., Tzahar, E., Liu, N., Wen, D. and Yarden, Y. (1993) EMBO J. **12**, 961–971
15 Wallasch, C., Weiss, F. U., Niederfellner, G., Jallal, B., Issing, W. and Ullrich, A. (1995) EMBO J. **14**, 4267–4275
16 Riese, II, D. J., van Raaij, T. M., Plowman, G. D., Andrews, G. C. and Stern, D. F. (1995) Mol. Cell. Biol. **15**, 5770–5776
17 Beerli, R. R., Graus-Porta, D., Woods-Cook, K., Chen, X. M., Yarden, Y. and Hynes, N. E. (1995) Mol. Cell. Biol. **15**, 6496–6505
18 Zhang, K., Sun, J. L., Liu, N. L., Wen, D. Z., Chang, D., Thomason, A. and Yoshinaga, S. K. (1996) J. Biol. Chem. **271**, 3884–3890
19 Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B. J., Sela, M. and Yarden, Y. (1996) EMBO J. **15**, 2452–2467
20 Pinkas-Kramarski, R., Shelly, M., Glathe, S., Ratzkin, B. J. and Yarden, Y. (1996) J. Biol. Chem. **271**, 19029–19032
21 Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J. and Yarden, Y. (1996) Mol. Cell. Biol. **16**, 5276–5287
22 Carraway, III, K. L. and Cantley, L. C. (1994) Cell **78**, 5–8
23 Lemmon, M. A. and Schlessinger, J. (1994) Trends Biochem. Sci. **19**, 459–463
24 Hynes, N. E. and Stern, D. F. (1994) Biochim. Biophys. Acta Rev. Cancer **1198**, 165–184
25 Cheng, K. R. and Koland, J. G. (1996) J. Biol. Chem. **271**, 311–318
26 Koland, J. G., O'Brien, K. M. and Cerione, R. A. (1990) Biochem. Biophys. Res. Commun. **166**, 90–100
27 Hellyer, N. J., Kim, H.-H., Graves, C. H., Sierke, S. L. and Koland, J. G. (1995) Gene **165**, 279–284
28 Clark, A. J. L., Beguinot, L., Ishii, S., Ma, D. P., Roe, B. A., Merlino, G. T. and Pastan, I. (1986) Biochim. Biophys. Acta **867**, 244–251
29 Guy, P. M., Carraway, III, K. L. and Cerione, R. A. (1992) J. Biol. Chem. **267**, 13851–13856

30   Summers, M. D. and Smith, G. E. (1987) A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agriculture Experimental Station, College Station, Texas

31   Fung, M.-C., Chiu, K. Y. M., Weber, T., Chang, T.-W. and Chang, N. T. (1988) J. Virol. Methods **19**, 33–42

32   Bradford, M. (1976) Anal. Biochem. **72**, 248–254

33   Laemmli, U. K. (1970) Nature (London) **227**, 680–685

34   Nelder, J. A. and Mead, R. (1965) Comput. J. **7**, 308–313

35   Sreerama, N. and Woody, R. W. (1993) Anal. Biochem. **209**, 32–44

36   Wedegaertner, P. B. and Gill, G. N. (1989) J. Biol. Chem. **264**, 11346–11353

37   Koland, J. G. and Cerione, R. A. (1990) Biochim. Biophys. Acta **1052**, 489–498

38   Sierke, S. L. and Koland, J. G. (1993) Biochemistry **32**, 10102–10108

39   Cobb, M. H., Sang, B.-C., Gonzales, R., Goldsmith, E. and Ellis, L. (1989) J. Biol. Chem. **264**, 18701–18706

40   Herrera, R., Lebwohl, D., de Herreros, A. G., Kallen, R. G. and Rosen, O. M. (1988) J. Biol. Chem. **263**, 5560–5568

41   Wedegaertner, P. B. and Gill, G. N. (1992) Arch. Biochem. Biophys. **292**, 273–280

42   Wei, L., Hubbard, S. R., Hendrickson, W. A. and Ellis, L. (1995) J. Biol. Chem. **270**, 8122–8130

43   Selva, E., Raden, D. L. and Davis, R. J. (1993) J. Biol. Chem. **268**, 2250–2254

44   Coker, K. J., Staros, J. V. and Guyer, C. A. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 6967–6971

45   Hack, N., Sue-A-Quan, A., Mills, G. B. and Skorecki, K. L. (1993) J. Biol. Chem. **268**, 26441–26446

46   Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Leichleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B. and Cantley, L. C. (1993) Cell **72**, 767–778

ErbB3 (HER3) interaction with the p85 regulatory subunit of phosphoinositide 3-kinase

Nathan J. HELLYER, Kunrong CHENG[1] and John G. KOLAND[2]

Department of Pharmacology, University of Iowa College of Medicine, Iowa City, IA 52242, U.S.A.

ErbB3 (HER3), a unique member of the ErbB receptor family, lacks intrinsic protein tyrosine kinase activity and contains six Tyr-Xaa-Xaa-Met (YXXM) consensus binding sites for the SH2 domains of the p85 regulatory subunit of phosphoinositide 3-kinase. ErbB3 also has a proline-rich sequence that forms a consensus binding site for the SH3 domain of p85. Here we have investigated the interacting domains of ErbB3 and p85 by a unique application of the yeast two-hybrid system. A chimaeric ErbB3 molecule containing the epidermal growth factor receptor protein tyrosine kinase domain was developed so that the C-terminal domain of ErbB3 could become phosphorylated in the yeast system. We also generated several ErbB3 deletion and Tyr → Phe site-specific mutants, and observed that a single ErbB3 YXXM motif was necessary and sufficient for the association of ErbB3 with p85. The incorporation of multiple YXXM motifs into the ErbB3 C-terminus enabled a stronger ErbB3/p85 interaction. The proline-rich region of ErbB3 was not necessary for interaction with p85. However, either deletion or mutation of the p85 SH3 domain decreased the observed ErbB3/p85 association. Additionally an ErbB3/p85 SH3 domain interaction was detected by an assay *in vitro*. These results were consistent with a model in which pairs of phosphorylated ErbB3 YXXM motifs co-operate in binding to the tandem SH2 domains of p85. Although a contributing role for the p85 SH3 domain was suggested, the N- and C-terminal SH2 domains seemed to be primarily responsible for the high-affinity association of p85 and ErbB3.

INTRODUCTION

ErbB (HER) family receptors regulate mammalian cell survival, proliferation and differentiation in response to growth factors such as epidermal growth factor (EGF) and heregulin [1,2]. ErbB3 (HER3) is unique among ErbB family members in that it is devoid of intrinsic protein tyrosine kinase activity [3,4] as well as being the only family member that contains multiple consensus sites in its C-terminal domain for binding the p85 subunit of phosphoinositide (PI) 3-kinase. Although ErbB3 lacks kinase activity, Tyr residues within its cytoplasmic tail become phosphorylated on its heterodimerization with other ErbB family receptors, such as the EGF receptor (EGFR) or ErbB2, which allows ErbB3 to associate with PI 3-kinase. The EGF-dependent phosphorylation of ErbB3 triggers ErbB3/p85 association and increases the level of PI 3-kinase activity that is co-immuno-precipitated with ErbB3 [5]. Likewise, when ErbB3 is phosphorylated by EGFR *in vitro* and added to cell lysates, ErbB3 co-immunoprecipitates with both p85 and the catalytic activity of the p110 subunit of PI 3-kinase [6]. Additionally it has been shown that in mouse fibroblasts transfected with ErbB2 and ErbB3 cDNA species, PI 3-kinase activity and mitogenesis increase in a heregulin-dependent manner, with both responses being dependent on the presence of ErbB3 [7]. PI 3-kinase activation by the ErbB2/ErbB3 co-receptor complex might also mediate heregulin-induced proliferation in mammary epithelial cells [8].

The p85 regulatory subunit of PI 3-kinase contains five distinct domains: an SH3 domain, a Bcr-homology region, two SH2 domains, and an inter-SH2 (IS) domain. The IS domain has been shown to be the region of p85 responsible for high-affinity interaction with the p110 catalytic subunit of PI 3-kinase [9–13]. The proline-rich Bcr domain exhibits sequence similarity with the breakpoint cluster region gene product [14] and has been implicated in an intramolecular interaction with the p85 SH3 domain [15], as well as in mediating intermolecular associations with the SH3 domains of Src protein tyrosine kinase family members [16–19].

The amino acid sequence motif Tyr-Xaa-Xaa-Met (YXXM) has been shown, when phosphorylated, to specifically associate with the SH2 domains of p85 [20,21]. Six such consensus binding sites for the SH2 domains of p85 occur at the C-terminus of ErbB3; if phosphorylated, they could potentially mediate the association of ErbB3 and p85. Synthetic ErbB3-derived phospho-peptides that contain phosphorylated YXXM sequences have been shown to inhibit the association of p85 with ErbB3 in the context of a chimaeric EGFR/ErbB3 receptor [22]. However, it is not known which specific ErbB3 YXXM motifs are phosphorylated *in vivo*, and whether the C-terminal, the N-terminal, or both p85 SH2 domains account for p85 association with ErbB3.

The C-terminal tail of ErbB3 also contains a proline-rich sequence, PPRPPRP, that forms a consensus binding site for the SH3 domains of both p85 and Src-family protein tyrosine kinases [23–25] and could also contribute to the association of ErbB3 with p85. SH3 domains recognizing PXXP amino acid motifs in

signalling proteins have been shown to mediate protein–protein interactions independently as well as in co-operation with SH2 domains. Interestingly, it has been observed that the SH3 domain of p85 can interact with either of two proline-rich motifs [PPTPKPRPPRPLP (residues 84–96) and PAPALPPKPPKP (residues 303–314)] contained within the Bcr region of p85 itself [15]. This intramolecular interaction could cause conformational changes within p85 that regulate its association with other proteins or modulate the catalytic activity of the associated p110 catalytic subunit of PI 3-kinase [15].

The yeast two-hybrid system is a genetic approach for studying protein–protein interactions [26], which has been used to study the interaction between p85 and the insulin and insulin-like growth factor 1 receptors [27,28] as well as the interaction between the p85 and p110 subunits of PI 3-kinase [10]. Here we describe a novel application of the yeast two-hybrid system in mapping the interacting domains of p85 and ErbB3.

EXPERIMENTAL

Yeast strains and expression plasmids

The yeast two-hybrid system reporter strain SFY526 (MATα ura3-52 his3-Δ200 ade2-801 trp-901 leu2-3,112 can^r gal4-542 gal 80-538 URA3::GAL1-lacZ) and the respective Gal4 DNA-binding domain (pGBT9) and transcriptional activation domain (pGAD424) vectors have been described [26]. Various pGAD-p85 vectors [10] were kindly provided by the laboratory of Dr. Jeffrey Pessin (University of Iowa, Iowa City, IA, U.S.A.). The rat ErbB3 cDNA sequence has been described previously [29]. The cDNA encoding the protein tyrosine kinase domain of the human EGFR (amino acid residues 647–972) was excised from pMMTV-ER [30] and subcloned into the yeast two-hybrid expression vector pGBT9 to yield pGBT-EGFRΔCT. The cDNA encoding the C-terminal tail of ErbB3 (residues 938–1339) was subsequently subcloned in-frame with the EGFR protein tyrosine kinase domain sequence by use of an *Acc*I restriction site conserved in both EGFR and ErbB3 cDNA sequences to generate pGBT-ER/B3. pGBT-ER(K/M)/B3 was generated by subcloning into pGBT-ER/B3 a cDNA encoding an EGFR protein tyrosine kinase domain with a Lys-721 → Met mutation that abolished kinase activity [31]. pGBT-ER/B3Δ1121 was made by a *Sac*II/*Sal*I restriction digestion and subsequent religation of the plasmid. pGBT-ER/B3Δ1204 and pGBT-ER/B3Δ1237 were made by PCR amplification of truncated sequences by using the forward primer 5′-ATATATAGCGAGATCTCCTCC-3′ and the reverse primers 5′-ATGTCGACGCTACCCCTTCTCTT-3′ or 5′-ATGTCGACTCTCTGTGTACTGCC-3′ respectively. These primers incorporated *Bgl*II and *Sal*I restriction sites into the amplified sequences that allowed them to be subcloned in-frame with the pGBT-ER/B3 protein tyrosine kinase domain plasmid. The pGBT vector conveniently provides in-frame stop codons for all three deletion mutants. The pGBT-ER/B3-ΔPro, -1051Y, -1194Y, -1051/1194Y, -1051/1194F, -6F and pGAD-p85-D21N site mutants were generated bu using the ExSite mutagenesis method (Stratagene, La Jolla, CA, U.S.A.).

β-Galactosidase activity assays

Quantitative assays of β-galactosidase activity were performed as described by Fields and Song [26], except that Chlorophenol Red β-D-galactopyranoside (Sigma) was used as the chromogenic substrate and the absorbance was determined at 574 nm. One unit of β-galactosidase activity was defined as $(1000 \times A_{574})/[A_{600} \times \text{volume (ml)} \times \text{time (min)}]$ as described by Miller [32]. All

results are expressed as means ± S.E.M. for at least four independent assays performed on distinct colonies.

Immunoblotting

Yeast cells were grown in 50 ml of selective medium overnight; they were then pelleted, washed in 1 ml of water and resuspended in 250 μl of 20 % (w/v) trichloroacetic acid. Glass beads (500 μm) were added to the samples, which were then vortex-mixed six times for 30 s each time. The trichloroacetic acid precipitates were neutralized with 250 μl of acetone/ammonium hydroxide (5:0.3, v/v), after which 400 μl of 1 % (w/v) SDS was added and the samples were boiled for 7 min. Lysates were transferred to fresh tubes containing 100 μl of 5 × sample buffer, and 50 μl of each sample was subjected to SDS/PAGE. Resolved proteins were transferred to an Immobilon-P membrane (Millipore, Bedford, MA, U.S.A.), which was blocked with 5 % (w/v) dry milk in PBS then immunostained with ErbB3-specific (2C3; Neo-Markers, Fremont, CA, U.S.A.), GAL4(DBD)-specific (RK5C1, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) or p85 N-terminal SH2 domain-specific (Upstate Biotechnology, Lake Placid, NY, U.S.A.) antibodies. Membranes were incubated with horseradish peroxidase-conjugated anti-(mouse Ig) and detected by an enhanced chemiluminescence method (Amersham, Chicago, IL, U.S.A.).

Binding assays *in vitro*

Several cDNA species encoding N-terminal domains of p85 were amplified by PCR and expressed as glutathione S-transferase (GST) fusion proteins, which were purified as described previously [33]. An Asp-21 → Asn amino acid substitution was introduced into the SH3 domain by use of the ExSite mutagenesis method (Stratagene). Each GST or GST–p85 peptide (2 nmol) was incubated in buffer A [20 mM sodium Hepes (pH 7.4)/ 50 mM NaCl/10 % (v/v) glycerol] supplemented with 1.8 mM MnCl$_2$, 0.02 % (w/v) Triton X-100, and 2.0 pmol of purified recombinant ErbB3 cytosolic domain (hisTKD-B3) [4] for 15 min on ice (total volume 200 μl). The mixture was added to gluta-thione–agarose (1:1 suspension in buffer A) and incubated for 15 min at 4 °C. The agarose suspensions were centrifuged for 5 s at 600 *g*. The pellets were washed in 500 μl of ice-cold buffer A and then suspended in 110 μl of SDS/PAGE sample buffer. Samples (20 μl) were resolved by SDS/PAGE and subjected to immunoblotting with ErbB3 antibody. GST–p85 peptides (910 pmol) were also analysed by SDS/PAGE with Coomassie Blue staining.

RESULTS

Generation of a chimaeric yeast two-hybrid construct to examine the interaction between ErbB3 and p85

Analysis of the deduced amino acid sequence of the ErbB3 C-terminal phosphorylation domain reveals six consensus binding sites for the N-terminal and C-terminal SH2 domains of p85, as well as a proline-rich candidate ligand for the p85 SH3 domain. To examine the interaction between p85 and ErbB3 with the two-hybrid system, we generated a pGBT9 yeast two-hybrid vector expressing the cytosolic domain of ErbB3 (residues 660–1339). The ErbB3 cytosolic domain did not seem to interact with p85 (Figure 1), presumably owing to the lack of intrinsic kinase activity in ErbB3 and the absence of an associating kinase capable of phosphorylating ErbB3 in yeast. To overcome the

Figure 1   Interaction of an EGFR/ErbB3 chimaeric construct (ER/B3) with the p85 protein in the yeast two-hybrid system

The yeast reporter strain SFY526 was co-transformed with the indicated pGBT-receptor and pGAD-p85 plasmid constructs, and transformants were assayed for β-galactosidase reporter gene activity. The EGFR protein tyrosine kinase domain (ER TKD, residues 647–972) and ErbB3 cytosolic domain (residues 660–1339) are shown schematically. ER/B3 is a chimaera composed of the EGFR protein tyrosine kinase domain (residues 647–918) fused to the phosphorylation domain of the ErbB3 protein (residues 938–1339) and expressed via the pGBT vector. A conserved Lys residue in the EGFR protein tyrosine kinase domain essential for protein kinase activity was replaced with Met in the ER(K/M)/B3 construct. The pGAD-p85 construct expresses the full-length p85 subunit of PI 3-kinase as a GAD fusion protein [10]. Abbreviation: U, units.

lack of phosphorylation in the yeast system, we developed an EGFR/ErbB3 chimaeric construct (ER/B3) containing a functional EGFR protein tyrosine kinase domain in place of the inactive ErbB3 protein tyrosine kinase homology domain. In this construct, the ErbB3 C-terminus was fused to the end of the EGFR protein tyrosine kinase domain in a manner that preserved the conserved amino acid sequences at the domain boundary. By itself, the EGFR kinase domain (ER TKD, residues 647–972) was incapable of association with p85 (Figure 1). However, ER/B3 strongly associated with p85, apparently in a phosphory-

lation-dependent manner, as a kinase-deficient ER/B3 construct, ER(K/M)/B3, failed to interact with p85 (Figure 1).

To ensure that ER/B3 could not activate the *lacZ* reporter gene independently of p85, ER/B3 was transfected both alone and with a pGAD vector not containing a p85 cDNA insert. No activation of the lacZ reporter gene was observed in either case (results not shown). Similarly, the pGAD-p85 construct did not activate the reporter gene alone or in combination with an empty pGBT vector (results not shown). Western immunoblotting revealed that the GBT-fusion proteins were expressed at similar levels (see Figure 4A), which suggested that differences in protein interactions with p85 were not due to differences in protein expression levels. Taken together, these results demonstrated a specific interaction between ErbB3 and p85 within the yeast two-hybrid system.

Interaction between ER/B3 and p85 subdomains

To determine which regions of p85 associated with ErbB3, we tested the interaction of the SH3, Bcr and dual SH2 domains (n/cSH2) of p85 with ER/B3. The n/cSH2 domain exhibited the strongest interaction of the three domains. However, this interaction was less than half of that of full-length p85 (Figure 2). The Bcr and SH3 domains of p85 seemed to associate very weakly with ER/B3. Even though the SH3 domain of p85 exhibited a minimal interaction when tested alone, we suspected that the SH3 of p85 domain might be capable of a weak interaction with a proline-rich motif in ErbB3 and contribute to the maximally observed interaction between p85 and ErbB3, which seemed to be mediated predominantly by the two SH2 domains of p85. To test this hypothesis we generated a mutant p85 protein (p85-D21N) with an Asp → Asn substitution at Asp-21, which occurs within the SH3 domain of p85. Other groups have shown that this Asp → Asn mutation in the p85 SH3 domain disrupts the specific association between the SH3 domain and proline-rich recognition sequences [24,25]. We found that
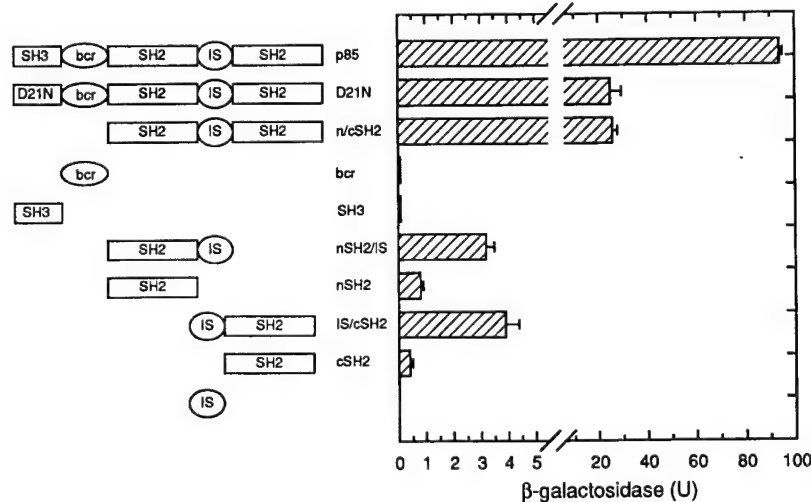
Figure 2   Interaction between ErbB3 and domains of the p85 protein

The yeast reporter strain SFY526 was co-transformed with pGBT-ER/B3 and the indicated pGAD-p85 plasmid constructs. p85-D21N has an Asp-21 → Asn substitution in the SH3 domain of p85. Distinct constructs expressing individual p85 domains as GAD fusion proteins are indicated: n/cSH2 (residues 330–720), Bcr (residues 77–353), SH3 (residues 9–87), nSH2/IS (residues 330–621), nSH2 (residues 330–434), IS/cSH2 (residues 429–720), cSH2 (residues 621–720), and IS (residues 429–621). Abbreviation: U, units.
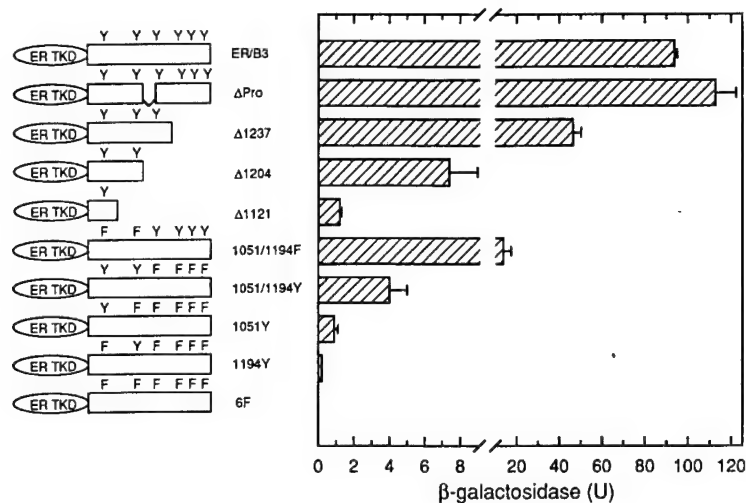
Figure 3   Interaction of C-terminal ER/B3 truncation mutants and Tyr → Phe site-specific mutants with p85

The yeast reporter strain SFY526 was co-transformed with pGAD-p85 and the indicated pGBT-ER/B3 plasmid constructs. Various ER/B3 C-terminal truncation mutants are shown with remaining YXXM motifs indicated. Various ER/B3 site-specific mutants are shown with indicated Tyr → Phe substitutions occurring in consensus sequences (YXXM → FXXM). ER/B3ΔPro corresponds to the deletion of a proline-rich consensus p85 SH3 domain-binding motif (residues 1205-1211). Abbreviation: U, units.
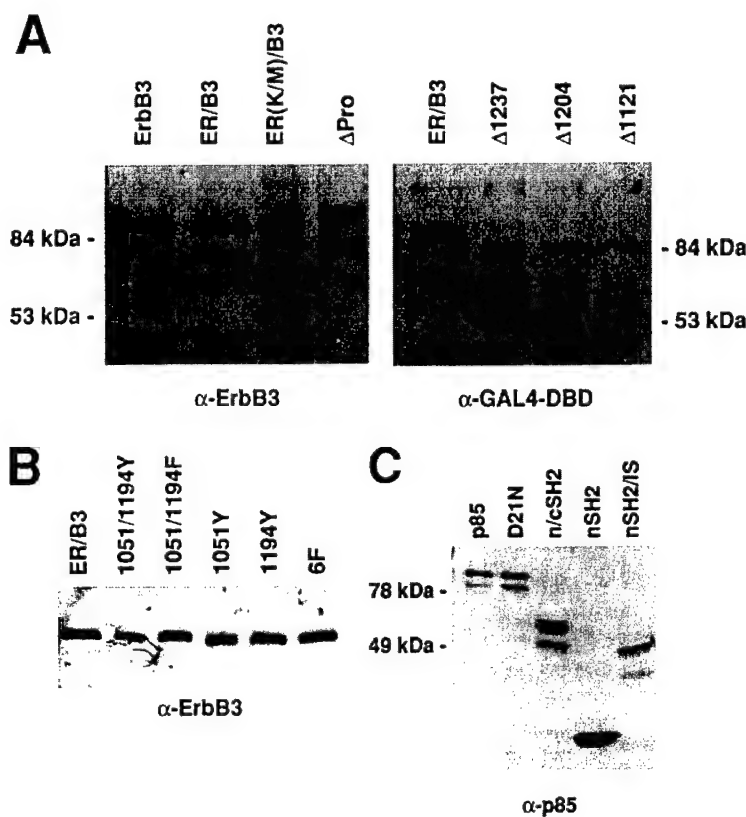
Figure 4   Immunoblotting of GBT and GAD fusion proteins

Lysates of yeast transfected with pGBT and pGAD expression vectors were immunoblotted to detect the expression of the yeast two-hybrid fusion proteins. (**A**) Left panel: detection of the approx. 90 kDa GBT-fusion proteins with an antibody recognizing the C-terminus of ErbB3. Right panel: detection of wild-type GBT-ErbB3 (approx. 90 kDa) and ER/B3 truncation mutants (approx. 75, 65 and 50 kDa) with an antibody specific for the Gal4 DNA-binding domain. (**B**) Detection of ER/B3 Tyr → Phe site mutants. (**C**) Detection of GAD-p85 constructs with an antibody recognizing the N-terminal SH2 domain of p85.

A

104 -
82 -

48 -

B

97 -
66 -
45 -
31 -
21 -
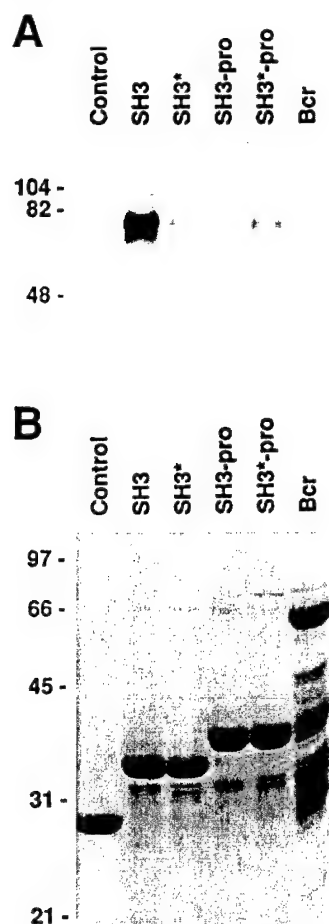
Figure 5   Association *in vitro* of GST–p85-SH3 domain fusion proteins with ErbB3

To investigate the role of the p85 SH3 domain in ErbB3/p85 interactions, GST fusion proteins containing either the p85 SH3 domain (GST–SH3, residues 1–80), the mutant SH3-D21N domain (GST–SH3*), the SH3 domain with a small proline-rich region of the Bcr domain appended (GST–SH3-pro, residues 1–101), or the contiguous SH3 and Bcr domains (GST–SH3-bcr, residues 1–339) were generated. (A) GST–p85 fusion proteins (2.0 nmol of each) were incubated with a recombinant ErbB3 cytosolic domain protein (2.0 pmol) and samples of glutathione-agarose precipitates were subsequently resolved by SDS/PAGE. Association of the ErbB3 protein with precipitated GST or GST–p85 fusion proteins was detected by immunoblotting with an ErbB3-specific antibody. (B) GST and GST–p85 fusion proteins (0.9 nmol of each) analysed by SDS/PAGE with Coomassie Blue staining.

p85-D21N exhibited a weaker association than wild-type p85 with ER/B3. Interestingly, the p85-D21N and n/cSH2 domain associations with the ER/B3 construct were comparable. The p85-D21N and n/cSH2 domain proteins were expressed at a level similar to or higher than wild-type p85 (see Figure 4C). These results suggested that the tandem SH2 domains of p85 are predominantly responsible for the observed ErbB3/p85 interaction but also suggested that the SH3 domain might have a role in the association of p85 with ErbB3.

Association of the N-terminal and C-terminal SH2 domains of p85 with ER/B3

We next tested whether both SH2 domains were required for the binding of p85 to ErbB3. The individual N-terminal (nSH2) and

C-terminal (cSH2) SH2 domains were capable of binding to ErbB3, with or without the IS domain (Figure 2). Inclusion of the IS domain increased the interaction between either nSH2 or cSH2 and ErbB3, perhaps by altering the conformation of the SH2 domains, as the IS domain alone did not interact with ErbB3 (Figure 2). The independent SH2 domains, however, bound much more weakly to ER/B3 than the dual SH2 domain, although comparable expression of these proteins was observed (see Figure 4C). This suggested that the tandem SH2 domains might participate in the ErbB3/p85 interaction via multiple ErbB3 phosphorylation sites.

Role of the ErbB3 proline-rich sequence in the association of p85

To test whether the proline-rich sequence motif in ErbB3 interacted with the SH3 domain of p85, a mutant ER/B3 construct (ER/B3ΔPro) was generated that lacked the PPRPPRP sequence (residues 1205 to 1211). Surprisingly, ER/B3ΔPro exhibited a slightly stronger interaction than ER/B3 with p85 (Figure 3). This result indicated that any potential interaction of the p85 SH3 domain with the PPRPPRP sequence did not significantly enhance the affinity of the ErbB3/p85 interaction. It is possible that the SH3 domain interacted with a sequence of ErbB3 other than the PPRPPRP sequence deleted. Alternatively, a competing intramolecular association of the SH3 domain with proline-rich sequences within the Bcr domain of p85 might have neutralized the effect of an ErbB3/SH3 interaction (see the Discussion section).

Association of p85 with ER/B3 C-terminally truncated mutants and Tyr → Phe site-specific mutants

We also generated several ER/B3-derived constructs with truncations of the ErbB3 C-terminal domain and the corresponding loss of YXXM motifs from the ErbB3 sequence (Figure 3). One truncated mutant (ER/B3Δ1237) deleted the three most C-terminal YXXM motifs containing Tyr-1257, Tyr-1273 and Tyr-1286. ER/B3Δ1237 mediated a weaker association with p85 than was observed with ER/B3. A more pronounced effect was observed with a truncation (ER/B3Δ1204) that removed an additional YXXM sequence corresponding to the loss of Tyr-1219. A third truncation, which resulted in the loss of Tyr-1194 and left only one YXXM motif (Tyr-1051) in the C-terminal domain of ErbB3, produced ER/B3Δ1121; this associated very weakly with p85. Because the accumulated loss of YXXM motifs correlated with progressively weakened associations between ER/B3 and p85, these results suggested that multiple YXXM motifs in ER/B3 contributed to the association with p85.

To study the roles of YXXM motifs further, we generated a mutant ErbB3 protein (ER/B3-6F) that contained Tyr → Phe substitutions at all six YXXM consensus sites. ER/B3-6F was unable to interact with p85, as expected (Figure 3). Consequently we constructed several YXXM add-back mutants. Here individual Tyr residues previously mutated to Phe in ER/B3-6F were restored to Tyr. Interestingly, the ER/B3-1051Y and ER/B3-1194Y add-back mutants, which contained single YXXM consensus sites for p85 binding, were able to interact with p85, albeit extremely weakly when compared with ER/B3. Furthermore the interaction with p85 was considerably augmented when two YXXM motifs were present in ER/B3, as was observed with the double add-back mutant ER/B3-1051/1194Y. These sites were not unique in interacting with p85 because a double mutant, ER/B3-1051/1194F, which contained Phe residues substituted for Tyr at positions 1051 and 1194, but retained the four C-terminal YXXM consensus sites, was able to mediate a stronger interaction than did ER/B3-1051/1194Y. In fact, as

suggested by analysis of the deletion mutants, it seemed that the larger the number of YXXM sites present in ER/B3, the stronger the interaction was with p85, regardless of the location of the sites. It also seemed from an examination of ER/B3-6F that at least one YXXM motif was required for binding and that other Tyr residues in ErbB3, which occur in consensus binding sites for other SH2 domain-containing proteins [29], could not substitute. Additionally, it seemed that multiple YXXM motifs co-operated in p85 binding, because add-back mutants containing multiple motifs associated in a more than additive manner compared with single-site add-back mutants. The mutant proteins showed expression levels similiar to that of ER/B3 (Figures 4A and 4B).

Association *in vitro* of the p85 SH3 domain with ErbB3

To investigate further the role of the p85 SH3 domain in interactions with ErbB3, we generated GST–p85 fusion proteins containing either the p85 SH3 domain (GST–SH3), an SH3 domain carrying the Asp-21 → Asn substitution (GST–SH3*), an SH3 domain with a small proline-rich sequence of the Bcr domain appended (GST–SH3-pro), or the contiguous SH3 and Bcr domains (GST–SH3-bcr) (see Figure 5). GST or GST–p85 fusion proteins were incubated with a recombinant ErbB3 cytosolic domain protein, after which the GST fusion proteins were precipitated with glutathione–agarose. ErbB3 that associated with the GST fusion proteins was detected by SDS/PAGE and Western immunoblotting. Indeed, an interaction was detected between GST–SH3 and ErbB3 (Figure 5), although this interaction was much weaker than that observed with the full-length GST–p85 protein and phosphorylated ErbB3 (results not shown). In contrast, the interaction of the mutant GST–SH3* with ErbB3 was almost undetectable. Interaction of GST–SH3-pro and GST–SH3-bcr with ErbB3 was also much weaker than that of GST–SH3, which suggested that the proline-rich sequences contained in p85 might compete in SH3 domain binding with the proline-rich sequence found in ErbB3.

DISCUSSION

The results of this study show that the association of ErbB3 with the p85 subunit of PI 3-kinase is mediated predominantly by interactions beteween the SH2 domains of p85 and YXXM motifs within the C-terminus of ErbB3, as previously suggested [22]. Other potential Tyr-phosphorylation motifs in the ErbB3 C-terminus do not seem to substitute for the conserved YXXM consensus p85 binding sites, in contrast with one study in which low-stringency protein–protein interactions were observed between EGFR phosphotyrosine sites and SH2 domain-containing proteins [34]. Our results also suggest that multiple Tyr residues within YXXM motifs can interact with the tandem SH2 domains of p85. Spatial relations between these Tyr residues could in principle determine optimal p85 binding [35]. Tyr-1257, Tyr-1273 and Tyr-1286 are separated by fewer than 20 residues each and could be ideally spaced for associating in concert with tandem SH2 domains [35]. Tyr-1051 and Tyr-1194, however, are separated by 142 residues and, from our results, still seemed to co-operate in binding p85. This suggests flexibility in the binding of p85 tandem SH2 domains to differentially spaced Tyr residues as hypothesized [35], in contrast with the relatively rigid spatial requirement of the tandem SH2 domains of SHP-2 [36] and ZAP-70 [37]. A tandem interaction of the SH2 domain with ErbB3 might optimize PI 3-kinase activation by ErbB3. It has been observed that bisphosphopeptides are more potent in stimulating PI 3-kinase activity when compared with mono-phosphopeptides [38–40].

Differential phosphorylation of Tyr residues in YXXM consensus sites within the ErbB3 C-terminus could ultimately determine which of these sites interact with p85. Little is currently known about the stoichiometry of phosphorylation of these various Tyr residues. Co-operation of the tandem SH2 domains of p85 in high-affinity binding to ErbB3, as we have apparently observed here (see Figures 2 and 3), would require multisite phosphorylation of ErbB3 molecules. However, we have not independently determined which ErbB3 Tyr residues were phosphorylated in the context of the yeast two-hybrid system, or whether individual ErbB3 molecules were phosphorylated on multiple Tyr residues. It is therefore possible that particular YXXM sites were predominantly phosphorylated and responsible for strong ErbB3 associations with p85, or alternatively that Tyr phosphorylation was distributed relatively equally between the YXXM sites. Our preliminary studies of COS7 cells transfected with ErbB3 mutants containing single YXXM sites have shown that p85 is capable of associating with any of the YXXM sites occurring in the ErbB3 C-terminus, which indicates that each of the YXXM sites in the ErbB3 C-terminus is potentially phosphorylated *in vivo* (N. J. Hellyer and J. G. Koland, unpublished work).

The presence of a consensus SH3 domain-binding motif in ErbB3 led us to investigate the roles of this motif and the p85 SH3 domain in ErbB3/p85 interactions. Deletion of the p85 SH3 domain or substitution of an SH3 domain residue critical for peptide recognition significantly decreased the interaction of ER/B3 and p85 (Figure 2). However, a similar effect was observed when examining the interaction of p85 with the insulin receptor cytosolic domain in the yeast system (results not shown), although the insulin receptor possesses no obvious candidate SH3 domain-binding sequences within its cytoplasmic domain. In addition, deletion of the PPRPPRP candidate SH3 domain-binding motif of the ErbB3 C-terminus did not significantly alter the ER/B3 interaction with p85 (Figure 3). Together these results imply that the potential interaction between this ErbB3 proline-rich sequence and the p85 SH3 domain does not contribute significantly to the overall affinity of the ErbB3/p85 interaction.

The decrease in the apparent affinity of the ErbB3/p85 interaction observed on deletion or mutation of the p85 SH3 domain might have been a consequence of the self-association of the p85 SH3 domain and a proline-rich region within the Bcr region of p85 [15], an interaction that could modulate the affinity of p85 SH2 domains for phosphotyrosine-containing substrates. It would also be possible that ligation of p85 SH2 domains by phosphotyrosine-containing peptides could exert a reciprocal effect on the SH3 domain, possibly reversing its intramolecular association with the Bcr domain and enhancing its accessibility to other signalling molecules. In the Ras-Gap/Rho-Gap interaction, it seems that when the tandem SH2 domains of Ras-Gap bind to phosphorylated Tyr residues of Rho-Gap, the conformation of Ras-Gap changes such that there is a 100-fold increase in the accessibility of the target-binding surface of its SH3 domain [41].

Our experiments *in vitro* provided evidence for intramolecular p85 interactions, and furthermore suggested that interactions between the p85 SH3 domain and proline-rich Bcr domain sequences could negate any positive binding contribution attributed to interactions between the p85 SH3 domain and the proline-rich sequence of the ErbB3 (see Figure 5). Hence these results leave open the possibility that ErbB3/p85 SH3 domain interactions do occur and that these interactions are of regulatory significance. For example, the disruption of an intramolecular p85 Bcr/SH3 domain interaction by the ErbB3 proline-rich sequence could modulate the function of p85, perhaps by

regulating its binding to or activation of low-molecular-mass GTP binding proteins such as Cdc42Hs [15,42] or by regulation of the associated p110 catalytic domain.

Other investigators have shown that yeast two-hybrid fusion proteins can be phosphorylated within the yeast two-hybrid system when co-expressed with either the platelet-derived growth factor receptor or Src protein tyrosine kinase domains [43,44]. In those studies, protein tyrosine kinase domains were co-expressed to phosphorylate mammalian signalling proteins that otherwise would not be significantly phosphorylated in yeast cells. This technique allowed investigators to screen for target proteins that interact with phosphorylated Tyr motifs occurring in these signalling proteins. Similarly, our strategy, in which an active protein tyrosine kinase domain was substituted for the otherwise inactive kinase domain of ErbB3, provided us with a functional chimaeric EGFR/ErbB3 protein that was used in mapping the sites of interaction between ErbB3 and the p85 subunit of PI 3-kinase. This strategy could be generally applicable to the investigation of other protein–protein interactions mediated by phosphorylation of Tyr residues. The ER/B3 construct could also be used to screen for novel proteins that interact with ErbB3 by use of the yeast two-hybrid approach.

REFERENCES

1   Alroy, I. and Yarden, Y. (1997) FEBS Lett. **410**, 83–86
2   Burden, S. and Yarden, Y. (1997) Neuron **18**, 847–855
3   Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A. and Carraway, III, K. L. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 8132–8136
4   Sierke, S. L., Cheng, K., Kim, H.-H. and Koland, J. G. (1997) Biochem. J. **322**, 757–763
5   Soltoff, S. P., Carraway, III, K. L., Prigent, S. A., Gullick, W. G. and Cantley, L. C. (1994) Mol. Cell. Biol. **14**, 3550–3558
6   Kim, H.-H., Sierke, S. L. and Koland, J. G. (1994) J. Biol. Chem. **269**, 24747–24755
7   Carraway, III, K. L., Soltoff, S. P., Diamonti, A. J. and Cantley, L. C. (1995) J. Biol. Chem. **270**, 7111–7116
8   Ram, T. G. and Ethier, S. P. (1996) Cell. Growth Differ. **7**, 551–561
9   Klippel, A., Escobedo, J. A., Hirano, M. and Williams, L. T. (1994) Mol. Cell. Biol. **14**, 2675–2685
10 Holt, K. H., Olson, A. L., Moye-Rowley, W. S. and Pessin, J. E. (1994) Mol. Cell. Biol. **14**, 42–49
11 Dhand, R., Hara, K., Hiles, I., Bax, B., Gout, I., Panayotou, G., Fry, M. J., Yonezawa, K., Kasuga, M. and Waterfield, M. D. (1994) EMBO J. **13**, 511–521
12 Hu, P. and Schlessinger, J. (1994) Mol. Cell. Biol. **14**, 2577–2583
13 Klippel, A., Escobedo, J. A., Hu, Q. and Williams, L. T. (1993) Mol. Cell. Biol. **13**, 5560–5566
14 Heisterkamp, N., Stam, K., Groffen, J., de Klein, A. and Grosveld, G. (1985) Nature (London) **315**, 758–61
15 Kapeller, R., Prasad, K., Janssen, O., Hou, W., Schaffhausen, B. S., Rudd, C. E. and Cantley, L. C. (1994) J. Biol. Chem. **269**, 1927–1933
16 Prasad, K., Janssen, O., Kapeller, R., Raab, M., Cantley, L. C. and Rudd, C. E. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 7366–7370
17 Prasad, K., Kapeller, R., Janssen, O., Repke, H., Duke, C. J., Cantley, L. C. and Rudd, C. E. (1993) Mol. Cell. Biol. **13**, 7708–7717
18 Liu, X., Marengere, L., Koch, C. A. and Pawson, T. (1993) Mol. Cell. Biol. **13**, 5225–5232
19 Vogel, L. B. and Fujita, D. J. (1993) Mol. Cell. Biol. **13**, 7408–7417
20 McGlade, C. J., Ellis, C., Reedijk, M., Anderson, D., Mbamalu, G., Reith, A. D., Panayotou, G., End, P., Bernstein, A., Kazlauskas, A. et al. (1992) Mol. Cell. Biol. **12**, 991–997
21 Piccione, E., Case, R. D., Domchek, S. M., Hu, P., Chaudhuri, M., Backer, J. M., Schlessinger, J. and Shoelson, S. E. (1993) Biochemistry **32**, 3197–3202
22 Prigent, S. A. and Gullick, W. J. (1994) EMBO J. **13**, 2831–2841
23 Yu, H., Rosen, M. K., Shin, T. B., Seidel, D. C., Brugge, J. S. and Schreiber, S. L. (1992) Science **258**, 1665–1668
24 Yu, H., Chen, J. K., Feng, S., Dalgarno, D. C., Brauer, A. W. and Schreiber, S. L. (1994) Cell **76**, 933–945
25 Weng, Z. G., Rickles, R. J., Feng, S. B., Richard, S., Shaw, A. S., Schreiber, S. L. and Brugge, J. S. (1995) Mol. Cell. Biol. **15**, 5627–5634
26 Fields, S. and Song, O.-K. (1989) Nature (London) **340**, 245–247
27 Lamothe, B., Bucchini, D., Jami, J. and Joshi, R. L. (1995) FEBS Lett. **373**, 51–55
28 Tartare-Deckert, S., Murdaca, J., Sawka-Verhelle, D., Holt, K. H., Pessin, J. E. and Van Obberghen, E. (1996) Endocrinology (Baltimore) **137**, 1019–1024
29 Hellyer, N. J., Kim, H.-H., Greaves, C. H., Sierke, S. L. and Koland, J. G. (1995) Gene **165**, 279–284
30 Clark, A. J. L., Beguinot, L., Ishii, S., Ma, D. P., Roe, B. A., Merlino, G. T. and Pastan, I. (1986) Biochim. Biophys. Acta **867**, 244–251
31 Chen, W. S., Lazar, C. S., Poenie, M., Tsien, R. Y., Gill, G. N. and Rosenfeld, M. G. (1987) Nature (London) **328**, 820–823
32 Miller, J. H. (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
33 Koland, J. G., O'Brien, K. M. and Cerione, R. A. (1990) Biochem. Biophys. Res. Commun. **166**, 90–100
34 Soler, C., Beguinot, L. and Carpenter, G. (1994) J. Biol. Chem. **269**, 12320–12324
35 Ottinger, E. A., Botfield, M. C. and Shoelson, S. E. (1998) J. Biol. Chem. **273**, 729–735
36 Eck, M. J., Pluskey, S., Trueb, T., Harrison, S. C. and Shoelson, S. E. (1996) Nature (London) **379**, 277–280
37 Hatada, M. H., Lu, X., Laird, E. R., Green, J., Morgenstern, J. P., Lou, M., Marr, C. S., Phillips, T. B., Ram, M. K., Theriault, K. et al. (1995) Nature (London) **377**, 32–38
38 Carpenter, C. L., Auger, K. R., Chanudhuri, M., Yoakim, M., Schaffhausen, B., Shoelson, S. and Cantley, L. C. (1993) J. Biol. Chem. **268**, 9478–9483
39 Herbst, J. J., Andrews, G., Contillo, L., Lamphere, L., Gardner, J., Lienhard, G. E. and Gibbs, E. M. (1994) Biochemistry **33**, 9376–9381
40 Rordorf, N. T., Van, H. D., Chen, D., White, M. F. and Backer, J. M. (1995) J. Biol. Chem. **270**, 3662–3666
41 Hu, K. Q. and Settleman, J. (1997) EMBO J. **16**, 473–483
42 Zheng, Y., Bagrodia, S. and Cerione, R. A. (1994) J. Biol. Chem. **269**, 18727–18730
43 Lioubin, M. N., Algate, P. A., Tsai, S., Carlberg, K., Aebersold, A. and Rohrschneider, L. R. (1996) Genes Dev. **10**, 1084–1095
44 Keegan, K. and Cooper, J. A. (1996) Oncogene **12**, 1537–1544

Signal transduction by epidermal growth factor and heregulin via the kinase-deficient ErbB3 protein

Hong-Hee KIM[1], Ulka VIJAPURKAR, Nathan J. HELLYER, Dolores BRAVO and John G. KOLAND[2]

Department of Pharmacology, The University of Iowa, College of Medicine, Iowa City, IA 52242-1109, U.S.A.

The role of protein tyrosine kinase activity in ErbB3-mediated signal transduction was investigated. ErbB3 was phosphorylated *in vivo* in response to either heregulin (HRG) in cells expressing both ErbB3 and ErbB2, or epidermal growth factor (EGF) in cells expressing both ErbB3 and EGF receptor. A recombinant receptor protein (ErbB3-K/M, in which K/M stands for Lys → Met amino acid substitution) containing an inactivating mutation in the putative ATP-binding site was also phosphorylated in response to HRG and EGF. Both the wild-type ErbB3 and mutant ErbB3-K/M proteins transduced signals to phosphatidylinositol 3-kinase, Shc and mitogen-activated protein kinases. Separate kinase-inactivating mutations in the EGF receptor and ErbB2 proteins abolished ErbB3 phosphorylation and signal transduction activated by EGF and HRG respectively. Hence the protein tyrosine kinase activity necessary for growth factor signalling via the ErbB3 protein seems to be provided by coexpressed EGF and ErbB2 receptor proteins.

INTRODUCTION

The ErbB/HER subfamily of polypeptide growth factor receptors [1,2] comprises the well-characterized epidermal growth factor (EGF) receptor (ErbB1/HER1), the *neu* oncogene product (ErbB2/HER2), and the more recently identified ErbB3/HER3 and ErbB4/HER4 receptor proteins. Each of these receptors is predicted to consist of an extracellular ligand-binding domain, a membrane-spanning domain, a cytosolic protein tyrosine kinase (PTK) domain and a C-terminal phosphorylation domain. The occurrence of heterodimerization among ErbB/HER family receptors seems to enhance the complexity of their cellular signalling mechanisms (see [3]). For example, EGF challenge of cells coexpressing the EGF receptor (EGFR) and either ErbB2, ErbB3 or ErbB4 leads to the phosphorylation of both the EGFR and the associated ErbB family member [3–11]. Various polypeptides in the heregulin (HRG or neuregulin) family have been shown to bind with high affinity to the ErbB4 receptor protein [12,13], but they apparently also bind to ErbB3 [13,14] and an ErbB2/ErbB3 [15] co-receptor complex. Whereas HRG has been shown to activate the PTK activity of the ErbB4 protein [12], it does not seem to activate PTK activity in cells expressing the ErbB3 protein alone [15]. ErbB2 does not itself bind HRG but coexpression of ErbB2 and ErbB3 yields a high-affinity HRG co-receptor, the PTK of which is activated in response to HRG binding [15,16].

Experiments *in vitro* have indicated that the PTK activity of the ErbB3 protein is attenuated significantly relative to that of other ErbB/HER family members; this has been attributed to the occurrence of non-conservative amino acid substitutions in the predicted catalytic domain of ErbB3 [17,18]. However, the ErbB3 protein is known to be strongly phosphorylated in a variety of cellular contexts. For example, ErbB3 is constitutively phosphorylated on tyrosine residues in a subset of human breast cancer cell lines overexpressing this protein [19], and is phosphorylated in response to either EGF or HRG in cells coexpressing ErbB3 and the EGFR [7,8] or ErbB2 [15] respectively. If the ErbB3 protein is devoid of intrinsic PTK activity, its phosphorylation in the cellular context would be presumed to result from the action of an associated PTK, for example via a transphosphorylation by either the EGFR or ErbB2 PTK. However, it is also possible that the PTK of ErbB3 is masked under the conditions of previous experiments *in vitro* or is expressed only in the context of an activated co-receptor complex. Indeed, Kraus et al. [19] observed a ligand-stimulated PTK activity in the ErbB3 protein.

The ErbB3 receptor is unique among receptor PTKs in that the amino acid sequence of the C-terminal phosphorylation domain [20,21] contains six repeats of the motif Tyr-Xaa-Xaa-Met (YXXM), known to serve, when phosphorylated, as a binding site for the Src homology 2 (SH2) domains of the p85 subunit of phosphatidylinositol (PtdIns) 3-kinase [22,23]. In previous studies, chimaeric proteins composed of the extracellular ligand-binding domain of EGFR and the cytoplasmic domain of ErbB3 were used to examine the cellular proteins involved in ErbB3 signal transduction [24,25]. EGF stimulation of NIH-3T3 cells expressing these chimaeric proteins resulted in their increased phosphorylation on tyrosine residues and triggered their association with PtdIns 3-kinase. Subsequent investigations of cancer cells endogenously expressing both the EGFR and ErbB3 [7,8] and transfected fibroblasts ectopically expressing these proteins [26] showed EGF-dependent ErbB3 phosphorylation and ErbB3/PtdIns 3-kinase association. As the YXXM motif is not present in the C-terminal phosphorylation domain of the EGFR, it seems that ErbB3 might co-operate with EGFR in the activation of a mitogenic signalling pathway not otherwise directly engaged by the EGFR.

ErbB3, like other ErbB family receptor proteins [27], also incorporates a consensus motif, Asn-Pro-Xaa-Tyr (NPXY), for binding the PTB (phosphotyrosine-binding) domain of the Shc protein [28–32]. This motif has been implicated in the binding of Shc to EGFR and ErbB2 [28,33,34] and to the insulin receptor [35]. Phosphopeptide competition experiments have indicated that the sequence Asn-Pro-Asp-Tyr[1309] (NPDY) in human ErbB3, when phosphorylated, mediates the association of Shc with this receptor [25], a finding confirmed by our recent site-

directed mutagenesis experiments (U. Vijapurkar, K. Cheng and J. G. Koland, unpublished work). The Shc protein is rapidly phosphorylated on activation of various receptor or non-receptor PTKs and is involved in the translocation of the Grb2/Sos complex to the plasma membrane [36,37]. Sos, a Ras guanine nucleotide exchange factor, then activates the Ras protein [38,39], which in turn stimulates the phosphorylation and activation of mitogen-activated protein kinases (MAPKs or ERKs) through a protein kinase cascade [40–42].

The present study examined the role of receptor PTK activity in growth factor signal transduction mediated by the ErbB3 protein in conjunction with other ErbB family members. To this end, EGFR, ErbB2 and ErbB3 proteins, either alone or in combination, were expressed ectopically in cultured cell models. The importance of the intrinsic PTK activity of individual ErbB family members was assessed by the use of recombinant EGFR, ErbB2 and ErbB3 proteins incorporating kinase-inactivating mutations.

EXPERIMENTAL

Cell lines and reagents

NIH-3T3 and COS7 cells were purchased from the American Type Culture Collection and cultured as recommended. Recombinant HRG-β1 and antibodies recognizing ErbB2 (Ab-1) and ErbB3 (2F12, 2C3) were purchased from NeoMarkers. Phosphotyrosine-specific monoclonal antibody PY20 was obtained from Leinco Technologies and Transduction Laboratories. Shc, Grb2 and p85 antibodies and recombinant PY20 conjugated with horseradish peroxidase (RC20) were also acquired from Transduction Laboratories. EGFR-specific monoclonal antibody Ab4 and an MAPK-specific antibody were obtained from Oncogene Science and Zymed Laboratories respectively. Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents were purchased from Amersham. The expression plasmid containing wild-type rat ErbB3 cDNA, pcDNA3-ErbB3, is detailed elsewhere [43]. cDNA species encoding wild-type EGFR and the kinase-deficient EGFR-K/M (in which K/M stands for Lys → Met amino acid substitution) mutant protein were kindly provided by Dr. Gordon Gill (University of California, San Diego, CA, U.S.A.). The rat ErbB2 (Neu) expression plasmid was a gift from Dr. Robert Weinberg (Whitehead Institute, Cambridge, MA, U.S.A.).

Generation of ErbB3 and ErbB2 Lys → Met mutant proteins

Rat ErbB3 cDNA was mutated with the Transformer Site-directed Mutagenesis Kit (Clontech). A Met codon was introduced at amino acid position 740 to replace the Lys codon by using a 24 bp mutagenic primer, 5′-CAGTCTGCATTATGG-TCATGGAGG-3′. Lys^{758} of rat ErbB2 cDNA was mutated by use of the Ex-Site Mutagenesis kit (Stratagene) and a 24 bp mutagenic primer, 5′-CATCATGGTGTTGAGAGAAACA-C-3′. The affected regions of the ErbB2 and ErbB3 cDNA species were sequenced to verify that the mutant expression vectors had been constructed successfully.

Production of recombinant HRG-β1$^{177-241}$

The cDNA encoding the EGF-like domain of the β1 isoform of HRG (HRG-β1$^{177-241}$) was generated by reverse transcriptase-mediated PCR with the oligonucleotides 5′-GCGAATTCCC-ATCTTGTAAAATGTGCG-3′ (forward primer, corresponding to bp 623–641 of human HRG-β1 cDNA) and 5′-CTCGGCC-GCTACTCCGCCTCCATAAATTCAATC-3′ (reverse primer, corresponding to bp 794–815). The first-strand cDNA was

transcribed from human brain mRNA (Clontech) with the reverse primer and SuperscriptII reverse transcriptase (Gibco-BRL). PCR (30 cycles) was performed after addition of the forward primer and *Pfu* polymerase (Stratagene). The authenticity of the PCR-amplified HRG cDNA was verified by DNA sequencing, and the HRG cDNA was subcloned downstream of the glutathione S-transferase (GST) coding sequence in the expression vector pGEX-KG [44]. The GST–HRG fusion protein was purified from cultures of *Escherichia coli* by glutathione–agarose affinity chromatography, dialysed and then digested with thrombin for 30 min at room temperature. The majority of GST and undigested GST–HRG was removed by running the mixture through a second glutathione–agarose column. HRG was freeze-dried and further purified by Superose Fast Performance Liquid Chromatography (Pharmacia). Fractions containing HRG were identified by SDS/PAGE [45], pooled and stored at −85 °C. These manipulations resulted in the inclusion of nine amino acid residues, Gly-Ser-Pro-Gly-Asp-Pro-Pro-Ala-Asn, upstream of the authentic HRG peptide sequence. In some experiments a 30 kDa HRG-β1 protein (NeoMarkers) was employed, which corresponded to the secreted form of the growth factor. Although the potency of this protein was significantly greater than that of the HRG-β1$^{177-241}$ peptide, qualitatively similar results were obtained with the two HRG preparations.

Gene transfection, immunoprecipitation and immunoblotting

COS7 cells (10^7) were suspended in Dulbecco's modified Eagle's medium, electroporated at 250 V and 1180 μF (Cell-Porator; Gibco-BRL) in the presence of the indicated plasmids, and used for experiments at 36–60 h after transfection. NIH-3T3 cells were transfected by a calcium phosphate method [46], and clones expressing the ectopic genes were selected in the presence of geneticin (G418).

Dishes of cells (80–90 % confluent) were serum-starved for 16–20 h with cell culture medium containing 0.1 % (v/v) fetal calf serum. Cells were washed twice with serum-free medium and incubated with EGF or HRG diluted in culture medium containing 0.1 % BSA or the dilution vehicle for 5–7 min at 37 °C. Cells were washed twice with PBS and lysed with NP40 lysis buffer [1 % (v/v) Nonidet P40/50 mM Hepes/Na/150 mM NaCl/2 mM EDTA/3 mM EGTA/2 mM sodium orthovanadate/10 mM sodium pyrophosphate/50 mM NaF/2 μg/ml pepstatin A/10 μg/ml aprotinin/10 μg/ml leupeptin/2 mM PMSF (pH 7.4)]. The total cell lysate was centrifuged for 10 min at 13000 g and the supernatant was collected. Appropriate antibodies were added and incubated for 1 h on ice. Protein A–agarose or protein G–agarose was added and the suspensions were rocked for 1–3 h at 4 °C. Immunoprecipitates were washed with a buffer containing 1 % (v/v) Nonidet P40, 50 mM Hepes/Na, 150 mM NaCl, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 50 mM NaF, pH 7.4, and subjected to SDS/PAGE. Resolved proteins were transferred to a PVDF membrane and detected with the indicated antibodies by enhanced chemiluminescence luminography (Amersham). For the gel-shift assay of MAPK activation, the amount of bisacrylamide was reduced [acrylamide/bisacrylamide, 30:0.04 (w/v)] and the electrode buffer was used at double concentration.

RESULTS

EGF-dependent phosphorylation of ErbB3 in transfected cells expressing EGFR and ErbB3 proteins

Our previous studies have shown that the ErbB3 protein is an excellent substrate for the PTK activity of the EGFR [18].

A

EGFR: — WT K/M
ErbB3: — WT K/M WT K/M
EGF: - + - + - + + +

α-ER

α-B3

B

EGFR: — WT K/M
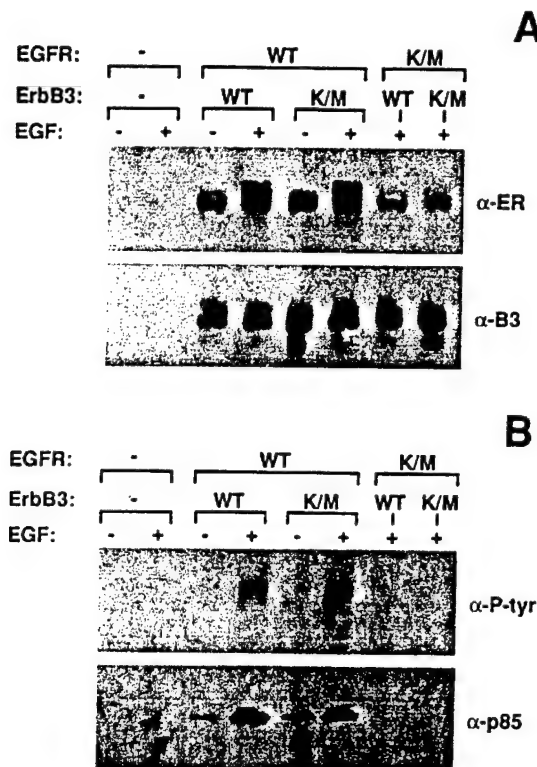ErbB3: — WT K/M WT K/M
EGF: - + - + - + + +

α-P-tyr

α-p85

Figure 1 EGF-dependent ErbB3 phosphorylation and ErbB3/PtdIns 3-kinase association in cells co-transfected with wild-type and mutant EGFR and ErbB3 cDNA species

COS7 cells were transfected by electroporation with wild-type (WT) EGFR, WT ErbB3, Lys^{721} → Met (K/M) mutant EGFR, or Lys^{740} → Met (K/M) mutant ErbB3 cDNA species as indicated. At 24 h after transfection, cells were serum-starved for 11 h and stimulated with 50 nM EGF or vehicle for 5 min at 37 °C. Detergent lysates of the cells were immunoprecipitated with ErbB3-specific antibody. (**A**) Expression of the recombinant ErbB3 protein in transfected cells was demonstrated by subjecting each immunoprecipitate to SDS/PAGE and immunoblotting with ErbB3-specific antibody (α-B3). EGFR expression was shown by immunoblotting an equivalent portion of each cell lysate with EGFR-specific antibody (α-ER). (**B**) Each immunoprecipitate was immunoblotted with anti-phosphotyrosine (α-P-tyr) and an antibody recognizing the p85 subunit of PtdIns 3-kinase (α-p85).

Evidence suggesting that the ErbB3 protein is a physiological substrate for EGFR has also been presented [7,8]. In these studies, cultured cells expressing high levels of both EGFR and ErbB3 were stimulated with EGF; phosphorylation of ErbB3 on tyrosine residues was detected by immunoblotting. To demonstrate more directly the cross-phosphorylation of ErbB3 by the EGFR, COS7 cells were co-transfected with EGFR and ErbB3 expression vectors. Both wild-type and mutant receptor proteins containing a kinase-inactivating Lys → Met substitution in their putative ATP-binding sites (EGFR-K/M [47] and ErbB3-K/M) were employed in these experiments.

Immunoblotting with receptor-specific antibodies indicated that each of the wild-type and K/M mutant receptors was successfully expressed in COS7 cells (Figure 1A). Cell lysates were subsequently immunoprecipitated with ErbB3 antibody; the immunoprecipitates were subjected to immunoblotting with anti-phosphotyrosine. In cells expressing wild-type receptors, EGF stimulated the phosphorylation of the ErbB3 protein (Figure 1B). Significantly, the mutant ErbB3-K/M protein was

phosphorylated on tyrosine residues to a similar extent to that of the wild-type protein. The ErbB3 immunoprecipitates showed no presence of EGFR when probed with an EGFR-specific antibody (results not shown). These results demonstrated that intrinsic ErbB3 PTK activity was not necessary for EGF-dependent ErbB3 phosphorylation. Activation of the EGFR also promoted the co-precipitation of p85 with both the wild-type and the mutant ErbB3 proteins (Figure 1B). In contrast, stimulation of the kinase-deficient form of the EGFR (EGFR-K/M) did not induce ErbB3 phosphorylation or ErbB3/p85 association. Therefore the activation of ErbB3 phosphorylation and signal transduction by EGF depended on the PTK activity of the EGFR but did not require that of ErbB3.

The apparent cross-phosphorylation of ErbB3 by the EGFR was not detected in parental COS7 cells, which indicated that the possible presence of endogenous EGFR and ErbB3 proteins in this cell line did not compromise the interpretation of these experiments (Figure 1B). Also, transfected COS7 cells over-expressing the ErbB3 proteins alone showed no phosphorylation of ErbB3 on stimulation with EGF (results not shown). These results were consistent with previous findings that the EGFR-mediated phosphorylation of ErbB3 occurs only in cells expressing a high level of EGFR [7,8].

HRG-dependent ErbB3 phosphorylation and ErbB3 signal transduction in cells expressing wild-type and mutant ErbB3 proteins

To extend our understanding of signal transduction mediated by the HRG-stimulated ErbB3 receptor, we generated stable NIH-3T3 cell lines that expressed a high level of wild-type ErbB3 and ErbB3-K/M (Figure 2A). Cells expressing the wild-type protein showed a constitutive phosphorylation of ErbB3 on tyrosine residues. However, as we have previously observed [43], phosphorylation of ErbB3 was clearly enhanced on stimulation with HRG-$\beta1^{177-241}$, a recombinant protein comprising the EGF-like domain of the HRG-$\beta1$ molecule. The mutant protein ErbB3-K/M was phosphorylated in response to HRG to a similar extent to the wild-type protein, which showed that intrinsic ErbB3 kinase activity was not required for HRG-dependent phosphorylation of the receptor. Treatment of mock-transfected cells with HRG did not elicit any phosphorylation response (Figure 2A). Also, ErbB3 immunoprecipitates showed no presence of the ErbB2 protein when probed with an ErbB2 antibody (results not shown). Given that ErbB3 seems not to possess intrinsic kinase activity [17,18], it was presumed that ErbB2, present endogenously in the NIH-3T3 fibroblasts, mediated the HRG-dependent phosphorylation of ErbB3. Our recent immunoblotting experiments have indicated that NIH-3T3 fibroblasts express the ErbB2 protein (U. Vijapurkar, K. Cheng and J. G. Koland, unpublished work), and when anti-phosphotyrosine immunoprecipitates were subjected to immunoblotting with ErbB2 antibody, HRG was seen to augment the phosphorylation of the endogenous ErbB2 protein (Figure 2A). These results suggested that the intrinsic kinase activity of ErbB2 might be responsible for the HRG-dependent phosphorylation of both ErbB2 and ErbB3, which was consistent with the notion that ErbB2 and ErbB3 functioned as HRG co-receptors.

To examine further the signalling potentials of the wild-type and mutant ErbB3 proteins, ErbB3 immunoprecipitates from transfected cells were probed with an antibody recognizing the p85 subunit of PtdIns 3-kinase. An HRG-dependent ErbB3/p85 association was seen with cells expressing either the wild-type or the mutant protein (Figure 2B). Apparently, mutation of the
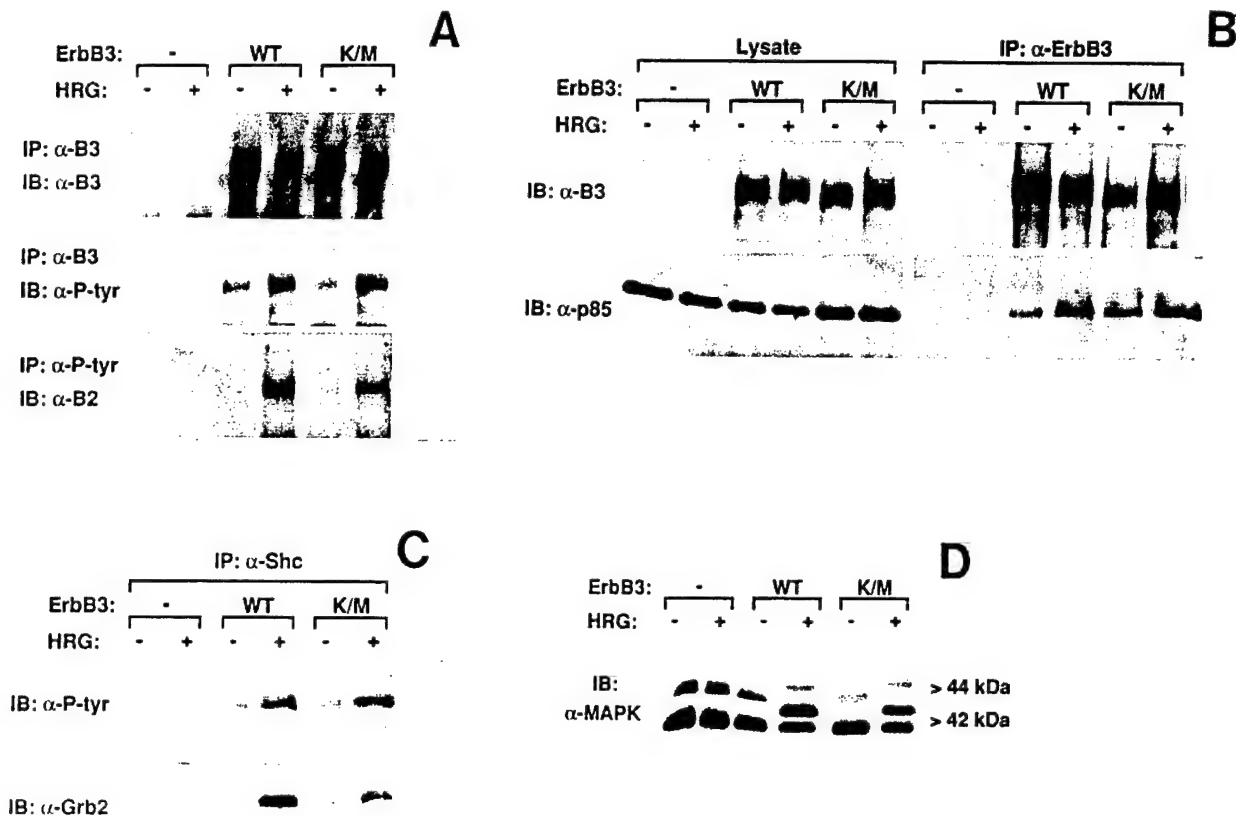
Figure 2   HRG-mediated phosphorylation and signal transduction of wild-type and mutant ErbB3 proteins in stably transfected NIH-3T3 cells

NIH-3T3 cells were transfected with either the parent pcDNA3 expression vector (—) or pcDNA3 vectors incorporating ErbB3-WT or ErbB3-K/M cDNA species as indicated. Cloned cells expressing comparable levels of wild-type (WT) and mutant proteins were selected and propagated. Mock-, ErbB3-WT- and ErbB3-K/M-transfected cells were serum-starved for 16 h and stimulated with either 30 nM HRG-$\beta1^{177-241}$ or vehicle for 5 min at 37 °C. (A) HRG-stimulated phosphorylation of ErbB3-WT, ErbB3-K/M, and endogenous ErbB2 proteins. HRG-stimulated or control cells were dispersed in NP40 lysis buffer. The lysates were immunoprecipitated (IP) with either ErbB3-specific antibody (α-B3) or anti-phosphotyrosine (α-P-tyr), and the immunoprecipitates were resolved by SDS/PAGE. ErbB3 immunoprecipitates were immunoblotted (IB) with either ErbB3 antibody or anti-phosphotyrosine as indicated. Anti-phosphotyrosine immunoprecipitates were immunoblotted with ErbB2-specific antibody (α-B2). (B) HRG-stimulated association of the p85 subunit of PtdIns 3-kinase with wild-type and mutant ErbB3 proteins. Lysates of HRG-stimulated or control cells were immunoprecipitated with ErbB3-specific antibody, and the immunoprecipitates were subjected to SDS/PAGE. Immunoprecipitation of the ErbB3 protein and HRG-stimulated co-immunoprecipitation of p85 were detected by immunoblotting with anti-ErbB3 (α-B3) and anti-p85 (α-p85) respectively. (C) Stimulation of Shc phosphorylation and Shc/Grb2 association by HRG. Cells treated as in (A) were lysed and immunoprecipitated with Shc-specific antibody. Immune complexes were immunoblotted with either the anti-phosphotyrosine–horseradish peroxidase conjugate RC20 (α-P-tyr) or a Grb2-specific antibody (α-Grb2). HRG-stimulated phosphorylation of the 52 kDa isoform of the Shc protein is indicated in the anti-phosphotyrosine blot. (D) HRG-stimulated activation of MAPK mediated by wild-type and mutant ErbB3 proteins. Cells were challenged with HRG or vehicle, then lysed as described above. Each cell lysate (30 μg of protein) was subjected to the gel mobility-shift assay of MAPK activation (see the Experimental section). Both the p42 and p44 forms of MAPK were observed in non-stimulated cells. The appearance of more slowly migrating bands indicated the activation of both MAPK forms in response to HRG.

ATP binding site of ErbB3, which would abolish any intrinsic kinase activity, did not affect its ability to associate with the p85 protein, an event presumably mediated by the HRG-dependent phosphorylation of the ErbB3 C-terminus.

The signal-transducing protein Shc has been implicated in the actions of a number of receptor PTKs [30,48–50]. In our recent studies (U. Vijapurkar, K. Cheng and J. G. Koland, unpublished work). Shc was found to associate with the ErbB3 protein and was phosphorylated on tyrosine residues in response to HRG. We sought to determine whether the HRG-stimulated kinase-inactive ErbB3-K/M would similarly involve Shc. Cells expressing either wild-type ErbB3 or the ErbB3-K/M mutant protein were stimulated with HRG-$\beta1^{177-241}$, and Shc immunoprecipitates were blotted with phosphotyrosine antibody. Shc was seen to be phosphorylated in response to HRG in cells expressing either protein (Figure 2C). Negligible Shc phosphorylation was detected in the mock-transfected NIH-3T3 cells. Among the three isoforms of Shc, phosphorylation of p52 was more evident than that of p61 or p46. Anti-Shc immunoprecipitates from the NIH-3T3 transfectants were also probed with a Grb2-specific antibody. HRG-$\beta1^{177-241}$ seemed to elicit a strong association of Grb2 and Shc in cells expressing ErbB3 (Figure 2C). Although the response of the mutant receptor protein was apparently weaker, ErbB3-K/M also mediated an HRG-dependent Shc/Grb2 association.

In the context of NIH-3T3 cells expressing wild-type ErbB3, MAPK isoforms undergo an HRG-dependent gel mobility shift (presumably owing to phosphorylation by MAPK kinase [51]), which corresponds to the activation of MAPK activity as indicated by immune complex kinase assays (U. Vijapurkar, K.
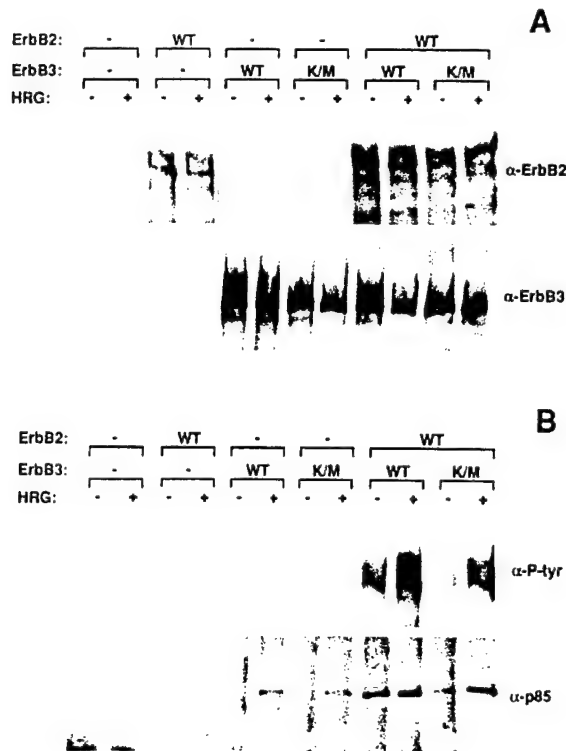
Figure 3   HRG-stimulated ErbB3 phosphorylation and ErbB3/PtdIns 3-kinase association in COS7 cells transfected with ErbB2 and ErbB3 cDNA species

(A) Coexpression of ErbB2 with wild-type (WT) and kinase-deficient ErbB3 proteins. COS7 cells were transiently transfected with the parent expression vector (—), or vectors incorporating cDNA species encoding ErbB2-WT, ErbB3-WT or ErbB3-K/M proteins as indicated. Transfected cells were serum-starved and then stimulated with 30 nM HRG-$\beta 1^{177-241}$ or vehicle for 7 min at 37 °C. Detergent lysates (1 mg of protein) were immunoprecipitated with ErbB3-specific antibody, and the immunoprecipitates were subjected to SDS/PAGE and immunoblotting with ErbB3 antibody (α-B3). The expression of ErbB2 was shown by immunoblotting 10 μg of cell lysate protein with ErbB2-specific antibody (α-B2). (B) HRG-stimulated ErbB3 phosphorylation and ErbB3/p85 association in cells expressing wild-type or mutant ErbB2 and ErbB3 proteins. The immunoprecipitates described in (A) were resolved by SDS/PAGE, and the phosphorylated forms of wild-type and mutant ErbB3 were detected by immunoblotting with anti-phosphotyrosine (α-P-tyr). Association of the p85 subunit of PtdIns 3-kinase with the ErbB3 proteins was detected by immunoblotting with p85 antibody (α-p85).

Figure 4   HRG-stimulated ErbB3 phosphorylation in cells co-transfected with wild-type and mutant ErbB2 and ErbB3 cDNA species

(A) COS7 cells were transiently transfected with vectors incorporating cDNA species of either wild-type (WT) or kinase-deficient Lys^{758} → Met (K/M) ErbB2 and either ErbB3 or ErbB3-K/M proteins as indicated. Cells were serum-starved and then stimulated with 1 nM recombinant HRG-$\beta 1$ (NeoMarkers) for 5 min at 37 °C. Detergent lysates (1.5 mg of protein) were immunoprecipitated with ErbB3 antibody, and each immunoprecipitate was subjected to SDS/PAGE and immunoblotting with ErbB3-specific antibody (α-B3). The expression of ErbB2 was shown by immunoblotting 10 μg of cell lysate protein with ErbB2-specific antibody (α-B2). (B) HRG-stimulated ErbB3 phosphorylation in cells coexpressing either ErbB2 or ErbB2-K/M and either ErbB3 or ErbB3-K/M. ErbB3 immunoprecipitates described in (A) were resolved by SDS/PAGE and immunoblotted with anti-phosphotyrosine (α-P-tyr).

Cheng and J. G. Koland, unpublished work). The gel mobility-shift assay [52] was used to determine whether HRG could stimulate MAPK phosphorylation via the ErbB3-K/M receptor protein. In the present study, when transfected cells were stimulated with HRG-$\beta 1^{177-241}$, both p42 (Erk2) and p44 (Erk1) forms of MAPK showed retarded migration on SDS/poly-acrylamide gels (Figure 2D). With respect to this MAPK gel mobility shift, wild-type and mutant ErbB3 proteins were in-distinguishable. The mock-transfected NIH-3T3 cells showed no HRG-dependent MAPK shift. These results confirmed that the Shc/Grb2/MAPK pathway, as well as the PtdIns 3-kinase pathway, was involved in HRG-stimulated signal transduction by ErbB3. Importantly, each of the signal transduction events mediated by the wild-type ErbB3 protein was also mediated by the ErbB3-K/M mutant. These results demonstrated that any intrinsic PTK activity of ErbB3 was not required for these signalling functions.
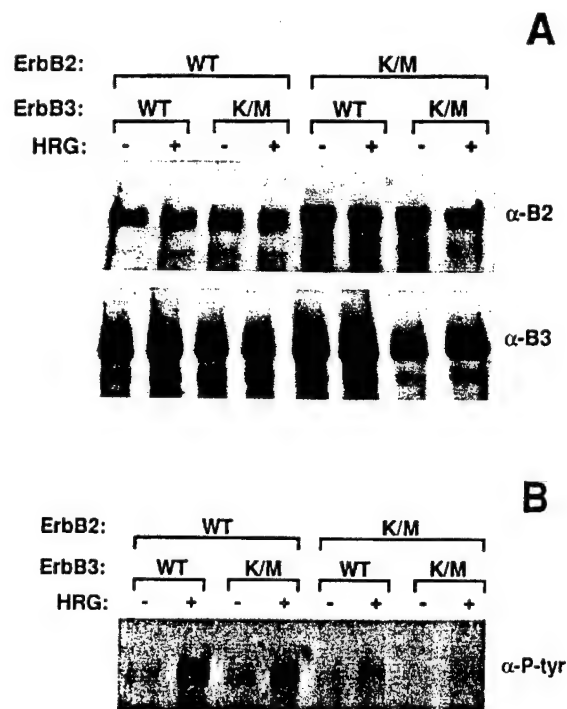
ErbB3 phosphorylation and ErbB3/PtdIns 3-kinase association in COS7 cells expressing ErbB2 and ErbB3 receptors

To investigate more directly the role of the ErbB2 receptor PTK in the HRG-stimulated phosphorylation of the ErbB3 protein, wild-type ErbB3 and ErbB3-K/M receptors were expressed alone or together with ErbB2 in COS7 cells (Figure 3A). When the transfected cells were stimulated with HRG, both wild-type and mutant ErbB3 proteins were phosphorylated on tyrosine residues (Figure 3B). ErbB3 phosphorylation was greatly enhanced by co-transfection of ErbB2, which indicated that ErbB3 was phos-phorylated by ErbB2 and/or an ErbB2-associated PTK. Again, negligible ErbB2 protein was detected in the ErbB3 immuno-precipitates (results not shown). The weak phosphorylation of the ErbB3 proteins in the cells transfected with ErbB3 cDNA species alone (Figure 3B) might have reflected the activity of a low level of endogenous ErbB2 present in these cells (U. Vijapurkar, K. Cheng and J. G. Koland, unpublished work). Both in cells transfected with ErbB3 cDNA alone and in cells co-transfected with ErbB2 cDNA, the phosphorylation of the ErbB3-K/M mutant protein was slightly less than that of the wild-type protein (see the Discussion section). ErbB3 immuno-precipitates from the transfected COS7 cells were also probed by

anti-p85 immunoblotting (Figure 3B). Association of the p85 subunit of PtdIns 3-kinase with both wild-type and mutant ErbB3 was found to be dependent on HRG-stimulation and was enhanced by the coexpression of ErbB2.

To underscore further the role of ErbB2 PTK activity in the HRG-stimulated phosphorylation of the ErbB3 protein, ErbB3 and ErbB3-K/M receptors were coexpressed with wild-type or Lys[758] → Met (K/M) mutant ErbB2 receptor (Figure 4A). When coexpressed with the wild-type ErbB2 protein, both wild-type ErbB3 and ErbB3-K/M were phosphorylated on tyrosine residues in response to HRG (Figure 4B). This phosphorylation was almost completely abolished when the wild-type or mutant ErbB3 protein was coexpressed with the ErbB2-K/M protein, which indicated that this phosphorylation was dependent on a functional ErbB2 PTK. The weak HRG-stimulated phosphorylation of ErbB3 and ErbB3-K/M in cells coexpressing the ErbB2-K/M protein was similar to that seen in COS7 cells transfected with ErbB3 cDNA species alone (compare Figure 3B).

DISCUSSION

The EGF-stimulated phosphorylation of ErbB3 has been demonstrated in studies of human cancer cell lines endogenously expressing EGFR and ErbB3 [7,8] and cultured cells ectopically expressing these receptors [11]. This apparent receptor cross-phosphorylation was confirmed here in studies of COS7 cells co-transfected with EGFR and ErbB3 cDNA species (Figure 1). Although it is possible that this phosphorylation was mediated by another protein kinase activated by the EGFR, the following observations suggest that the ErbB3 protein was phosphorylated directly by the EGFR. First, the kinase-deficient EGFR protein could not deliver this effect (Figure 1). Secondly, the cytoplasmic domain and C-terminal sequences of ErbB3 have previously been found to be excellent substrates for the EGFR [18,53]. Thirdly, evidence for cross-phosphorylation between other ErbB family receptors has accumulated [3–11,15,54–56].

The potential ErbB2-mediated phosphorylation of the ErbB3 protein was also investigated in the present study. The HRG-stimulated phosphorylation of ErbB3 expressed in COS7 cells was greatly enhanced with the coexpression of ErbB2 (Figure 3; see also [15]), and this effect was also observed with the ErbB3-K/M mutant protein. Coexpression of the kinase-deficient ErbB2-K/M protein with ErbB3 did not, however, significantly enhance HRG-dependent ErbB3 phosphorylation. These results indicated that ErbB3 kinase activity was not required for HRG-dependent ErbB3 phosphorylation, and that the PTK activity of ErbB2 was responsible for this phosphorylation. ErbB2/ErbB3 cross-phosphorylation would provide a mechanism by which both receptors could have important roles in HRG signalling. Whereas ErbB2 has intrinsic kinase activity, it cannot bind HRG in the absence of ErbB3 [15]. In contrast, ErbB3 binds HRG, but apparently does not possess intrinsic enzymic activity [17,18].

The phosphorylation of the ErbB3-K/M mutant protein on tyrosine residues was slightly lower than that of the wild-type protein when transfected COS7 cells were stimulated with HRG (Figures 3 and 4). This might suggest that ErbB3 does have intrinsic kinase activity and that the observed phosphorylation of ErbB3 was the sum of autophosphorylation and cross-phosphorylation by ErbB2. However, as we and others have not detected PTK activity in the ErbB3 protein [17,18], this possibility seems unlikely. Even if the ErbB3 receptor possessed protein kinase activity, the cross-phosphorylating activity of ErbB2 would seem to predominate over this activity, as the presence of ErbB2 greatly enhanced the phosphorylation of ErbB3. An

alternative explanation for the weaker HRG-dependent phosphorylation of the ErbB3-K/M mutant is the possibility that the Lys → Met substitution altered the conformation of the mutant protein such that it was less efficiently phosphorylated by ErbB2. Interestingly, no difference between the phosphorylation of ErbB3 wild-type and ErbB3-K/M was detected when the EGFR PTK mediated the cross-phosphorylation process (Figure 1).

The recruitment by ErbB3 of PtdIns 3-kinase to both EGF and HRG signalling pathways was also investigated here (Figures 1, 2 and 3). Both wild-type ErbB3 and the ErbB3-K/M mutant protein were shown to associate with the p85 subunit of PtdIns 3-kinase in a ligand-dependent manner. The presence of several YXXM motifs for p85 binding is unique to ErbB3, *Drosophila* insulin receptor [57] and the IRS-1 protein [58]; like IRS-1, ErbB3 seemed to function as a bridge between a receptor PTK and PtdIns 3-kinase. Previous studies have shown the HRG-dependent activation of PtdIns 3-kinase in NIH-3T3 cells transfected with both ErbB2 and ErbB3 cDNA species [26] and in cultured breast cancer cells endogenously expressing ErbB2 and ErbB3 [59].

At least one NPXY motif for Shc binding is present in each ErbB family protein. Graus-Porta et al. [60] reported that HRG induced the recruitment of Shc to ErbB family receptors and stimulated the phosphorylation of Shc on tyrosine residues in cells overexpressing both ErbB3 and ErbB4. HRG-stimulated phosphorylation of Shc has also been shown in cells over-expressing ErbB4 alone [61]. We have recently shown that ErbB3 can recruit the Shc protein on stimulation by HRG. This recruitment leads to the phosphorylation of Shc on tyrosine residues, the strong association of Shc and Grb2, and the activation of MAPKs (U. Vijapurkar, K. Cheng and J. G. Koland, unpublished work). Here we have demonstrated that the ErbB3-K/M mutant protein was also capable of mediating these signal transduction events. These results therefore suggest that any intrinsic PTK activity of ErbB3 is not necessary for its signalling function and that ErbB3, when phosphorylated in the presence of a kinase-active ErbB2 protein, can activate at least two distinct signalling pathways.

In summary, we have examined the role of PTK activity in growth factor signal transduction mediated by the ErbB3 protein. Whereas previous studies have shown that this protein exhibits negligible intrinsic PTK activity *in vitro* ([17,18], but see also [19]), the present results indicated that ErbB3 is catalytically inactive even in the context of a co-receptor complex. The ErbB3 protein is apparently phosphorylated by either the EGFR or ErbB2 PTK on stimulation of EGFR/ErbB3 or ErbB2/ErbB3 co-receptors with EGF or HRG respectively. Although the ErbB3 protein must rely on the PTK activity of another ErbB family member, it does uniquely participate in growth factor signalling by providing a high-affinity ligand-binding site in the case of the ErbB2/ErbB3 HRG co-receptor and by recruiting diverse intracellular signalling molecules. Phosphorylation of ErbB3 by a co-receptor partner triggers ErbB3/PtdIns 3-kinase association, Shc/Grb2 association and the activation of MAPKs. Given that the ErbB3 receptor protein is, along with other ErbB family members, often abundantly expressed in human cancer cells, the mitogenic signal transduction mechanisms investigated here might be particularly relevant in this context.

REFERENCES

1 Hynes, N. E. and Stern, D. F. (1994) Biochim. Biophys. Acta Rev. Cancer **1198**, 165–184

2 Carraway, III, K. L. and Burden, S. J. (1995) Curr. Opin. Neurobiol. **5**, 606–612

3 Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B. J., Sela, M. and Yarden, Y. (1996) EMBO J. **15**, 2452–2467

4 Stern, D. F. and Kamps, M. P. (1988) EMBO J. **7**, 995–1001

5 King, C. R., Borrello, I., Bellot, F., Comoglio, P. and Schlessinger, J. (1988) EMBO J. **7**, 1647–1651

6 Qian, X., Decker, S. J. and Greene, M. I. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 1330–1334

7 Soltoff, S. P., Carraway, III, K. L., Prigent, S. A., Gullick, W. G. and Cantley, L. C. (1994) Mol. Cell. Biol. **14**, 3550–3558

8 Kim, H.-H., Sierke, S. L. and Koland, J. G. (1994) J. Biol. Chem. **269**, 24747–24755

9 Karunagaran, D., Tzahar, E., Beerli, R. R., Chen, X. M., Graus-Porta, D., Ratzkin, B. J., Seger, R., Hynes, N. E. and Yarden, Y. (1996) EMBO J. **15**, 254–264

10 Zhang, K., Sun, J. L., Liu, N. L., Wen, D. Z., Chang, D., Thomason, A. and Yoshinaga, S. K. (1996) J. Biol. Chem. **271**, 3884–3890

11 Cohen, B. D., Kiener, P. A., Green, J. M., Foy, L., Fell, H. P. and Zhang, K. (1996) J. Biol. Chem. **271**, 30897–30903

12 Plowman, G. D., Green, J. M., Culouscou, J. M., Carlton, G. W., Rothwell, V. M. and Buckley, S. (1993) Nature (London) **366**, 473–475

13 Tzahar, E., Levkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D. and Yarden, Y. (1994) J. Biol. Chem. **269**, 25226–25233

14 Carraway, III, K. L., Sliwkowski, M. X., Akita, R., Platko, J. V., Guy, P. M., Nuijens, A., Diamonti, A. J., Vandlen, R. L., Cantley, L. C. and Cerione, R. A. (1994) J. Biol. Chem. **269**, 14303–14306

15 Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L. and Carraway, III, K. L. (1994) J. Biol. Chem. **269**, 14661–14665

16 Horan, T., Wen, J., Arakawa, T., Liu, N. L., Brankow, D., Hu, S., Ratzkin, B. and Philo, J. S. (1995) J. Biol. Chem. **270**, 24604–24608

17 Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A. and Carraway, III, K. L. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 8132–8136

18 Sierke, S. L., Cheng, K., Kim, H.-H. and Koland, J. G. (1997) Biochem. J. **322**, 757–763

19 Kraus, M. H., Fedi, P., Starks, V., Muraro, R. and Aaronson, S. A. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 2900–2904

20 Kraus, M. H., Issing, W., Miki, T., Popescu, N. C. and Aaronson, S. A. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 9193–9197

21 Plowman, G. D., Whitney, G. S., Neubauer, M. G., Green, J. M., McDonald, V. L., Todaro, G. J. and Shoyab, M. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 4905–4909

22 Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R. and Soltoff, S. (1991) Cell **64**, 281–302

23 Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J. et al. (1993) Cell **72**, 767–778

24 Fedi, P., Pierce, J. H., Di Fiore, P. P. and Kraus, M. H. (1994) Mol. Cell. Biol. **14**, 492–500

25 Prigent, S. A. and Gullick, W. J. (1994) EMBO J. **13**, 2831–2841

26 Carraway, III, K. L., Soltoff, S. P., Diamonti, A. J. and Cantley, L. C. (1995) J. Biol. Chem. **270**, 7111–7116

27 Plowman, G. D., Culouscou, J. M., Whitney, G. S., Green, J. M., Carlton, G. W., Foy, L., Neubauer, M. G. and Shoyab, M. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 1746–1750

28 Kavanaugh, W. M., Turck, C. W. and Williams, L. T. (1995) Science **268**, 1177–1179

29 Campbell, K. S., Ogris, E., Burke, B., Su, W., Auger, K. R., Druker, B. J., Schaffhausen, B. S., Roberts, T. M. and Pallas, D. C. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 6344–6348

30 Obermeier, A., Lammers, R., Weismuller, K.-H., Jung, G., Schlessinger, J. and Ullrich, A. (1993) J. Biol. Chem. **268**, 22963–22966

31 Laminet, A. A., Apell, G., Conroy, L. and Kavanaugh, W. M. (1996) J. Biol. Chem. **271**, 264–269

32 Blaikie, P., Immanuel, D., Wu, J., Li, N., Yajnik, V. and Margolis, B. (1994) J. Biol. Chem. **269**, 32031–32034

33 Batzer, A. G., Blaikie, P., Nelson, K., Schlessinger, J. and Margolis, B. (1995) Mol. Cell. Biol. **15**, 4403–4409

34 Okabayashi, Y., Kido, Y., Okutani, T., Sugimoto, Y., Sakaguchi, K. and Kasuga, M. (1994) J. Biol. Chem. **269**, 18674–18678

35 Gustafson, T. A., He, W., Craparo, A., Schaub, C. D. and O'Neill, T. J. (1995) Mol. Cell. Biol. **15**, 2500–2508

36 Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P. G. et al. (1992) Nature (London) **360**, 689–692

37 Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T. and Bowtell, D. (1993) Nature (London) **363**, 83–85

38 Chardin, P., Camonis, J. H., Gale, N. W., Van Aelst, L., Schlessinger, J., Wigler, M. H. and Bar-Sagi, D. (1993) Science **260**, 1338–1343

39 Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M. and Weinberg, R. A. (1993) Nature (London) **363**, 45–51

40 de Vries-Smits, A. M. M., Burgering, B. M. T., Leevers, S. J., Marshall, C. J. and Bos, J. L. (1992) Nature (London) **357**, 602–604

41 Kyriakis, J. M., Force, T. L., Rapp, U. R., Bonventre, J. V. and Avruch, J. (1993) J. Biol. Chem. **268**, 16009–16019

42 Robbins, D. J., Zhen, E., Cheng, M., Xu, S., Ebert, D. and Cobb, M. H. (1994) Adv. Cancer Res. **63**, 93–116

43 Hellyer, N. J., Kim, H.-H., Greaves, C. H., Sierke, S. L. and Koland, J. G. (1995) Gene **165**, 279–284

44 Guan, K. L. and Dixon, J. E. (1991) Anal. Biochem. **192**, 262–267

45 Laemmli, U. K. (1970) Nature (London) **227**, 680–685

46 Chen, C. and Okayama, H. (1987) Mol. Cell. Biol. **7**, 2745–2752

47 Chen, W. S., Lazar, C. S., Poenie, M., Tsien, R. Y., Gill, G. N. and Rosenfeld, M. G. (1987) Nature (London) **328**, 820–823

48 Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T. and Pelicci, P. G. (1992) Cell **70**, 93–104

49 Yokote, K., Mori, S., Hansen, K., McGlade, J., Pawson, T. and Heldin, H. K. (1994) J. Biol. Chem. **269**, 15337–15343

50 Pronk, G. J., McGlade, J., Pelicci, G., Pawson, T. and Bos, J. L. (1993) J. Biol. Chem. **268**, 5748–5753

51 Seger, R. and Krebs, E. G. (1995) FASEB J. **9**, 726–735

52 Posada, J. and Cooper, J. A. (1992) Science **255**, 212–215

53 Sierke, S. L. and Koland, J. G. (1993) Biochemistry **32**, 10102–10108

54 Riese, II, D. J., van Raaij, T. M., Plowman, G. D., Andrews, G. C. and Stern, D. F. (1995) Mol. Cell. Biol. **15**, 5770–5776

55 Riese, II, D. J., Bermingham, Y., van Raaij, T. M., Buckley, S., Plowman, G. D. and Stern, D. F. (1996) Oncogene **12**, 345–353

56 Tzahar, E., Waterman, H., Chen, X., Levkowitz, G., Karunagaran, D., Lavi, S., Ratzkin, B. J. and Yarden, Y. (1996) Mol. Cell. Biol. **16**, 5276–5287

57 Ruan, Y., Chen, C., Cao, Y. and Garofalo, R. S. (1995) J. Biol. Chem. **270**, 4236–4243

58 Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J. and White, M. F. (1991) Nature (London) **352**, 73–77

59 Ram, T. G. and Ethier, S. P. (1996) Cell Growth Differ. **7**, 551–561

60 Graus-Porta, D., Beerli, R. R. and Hynes, N. E. (1995) Mol. Cell. Biol. **15**, 1182–1191

61 Culouscou, J.-M., Carlton, G. W. and Aruffo, A. (1995) J. Biol. Chem. **270**, 12857–12863

Mutation of a Shc Binding Site Tyrosine Residue in ErbB3/HER3 Blocks Heregulin-dependent Activation of Mitogen-activated Protein Kinase*

Ulka Vijapurkar, Kunrong Cheng‡, and John G. Koland§

From the Department of Pharmacology, the University of Iowa College of Medicine, Iowa City, Iowa 52242-1109

The ErbB2 and ErbB3 proteins together constitute a functional coreceptor for heregulin (neuregulin). Heregulin stimulates the phosphorylation of both coreceptor constituents and initiates a variety of other signaling events, which include phosphorylation of the Shc protein. The role of Shc in heregulin-stimulated signal transduction through the ErbB2·ErbB3 coreceptor was investigated here. Heregulin was found to promote ErbB3/Shc association in NIH-3T3 cells expressing endogenous ErbB2 and recombinant ErbB3. A mutant ErbB3 protein was generated in which Tyr-1325 in a consensus Shc phosphotyrosine-binding domain recognition site was mutated to Phe (ErbB3-Y/F). This mutation abolished the association of Shc with ErbB3 and blocked the activation of mitogen-activated protein kinase by heregulin. Whereas heregulin induced mitogenesis in NIH-3T3 cells transfected with wild-type ErbB3 cDNA, this mitogenic response was markedly attenuated in NIH-3T3 cells transfected with the ErbB3-Y/F cDNA. These results showed a specific interaction of Shc with the ErbB3 receptor protein and demonstrated the importance of this interaction in the activation of mitogenic responses by the ErbB2·ErbB3 heregulin coreceptor complex.

The ErbB3/HER3 receptor protein is a member of the ErbB/HER family of growth factor receptors (1), the prototype of which is the epidermal growth factor (EGF)[1] receptor (ErbB1/HER1). Like other members of this family, the ErbB3 protein consists of an extracellular ligand binding domain, a transmembrane domain, an intracellular protein tyrosine kinase domain, and a C-terminal phosphorylation domain. Human heregulins (2) or their rat counterparts, the Neu differentiation factors (3), have been identified as a family of ligands for this receptor. ErbB3 is unique among ErbB/HER family members in that it has an impaired protein tyrosine kinase activity, which has been attributed to the substitution of amino acid residues invariantly conserved in protein tyrosine kinases (4, 5). However, ErbB3 tyrosine residue phosphorylation is observed when ErbB3 is coexpressed with other ErbB family members, apparently through the formation of heterodimeric receptor complexes (6, 7). Cells coexpressing EGF receptor and ErbB3 show an EGF-dependent ErbB3 phosphorylation (8, 9). Heregulin-stimulated phosphorylation of both ErbB2 and ErbB3 occurs in cells coexpressing these proteins (10–12), and although ErbB2 itself does not bind heregulin, ErbB2 and ErbB3 cooperate in the formation of a high affinity heregulin coreceptor complex (10). In addition, heregulin-dependent phosphorylation of EGF receptor and ErbB2 has been attributed to cross-phosphorylation by the kinase-intact heregulin receptor ErbB4 (13, 14).

Among the heterodimers formed within the ErbB family, the ErbB2·ErbB3 coreceptor complex is believed to elicit the most potent mitogenic signal (7, 11, 15). The contribution of ErbB3 to the mitogenic potential of ErbB family coreceptors might be enhanced by its unique C-terminal phosphorylation domain, which possesses several consensus sequences for the binding of signal-transducing proteins, including phosphoinositide (PI) 3-kinase, Grb2, Shc, SH-PTP2, and Src family protein tyrosine kinases (16). Notably, this domain contains six repeats of the consensus motif, Tyr-Xaa-Xaa-Met (YXXM), for binding to the p85 subunit of PI 3-kinase (17, 18). The role of PI 3-kinase in signal transduction by ErbB family coreceptors has begun to be clarified. The EGF-dependent association of PI 3-kinase with the ErbB3 protein has been observed in cancer cells expressing high levels of both EGF receptor and ErbB3 (8, 9). Also, a heregulin-dependent association of PI 3-kinase with ErbB3 has been seen in the context of the ErbB2·ErbB3 coreceptor, and the resulting activation of PI 3-kinase has been shown to be important for heregulin-stimulated mitogenesis (11).

Like other ErbB family members, the ErbB3 protein incorporates a consensus motif, Asn-Pro-Xaa-Tyr (NPXY), for binding to the Shc protein. Shc is an adapter protein that contains a C-terminal SH2 domain and an N-terminal phosphotyrosine-binding domain. The phosphotyrosine-binding domain of Shc specifically binds to phosphotyrosine in the NPXY sequence context (19–22) and mediates the binding of Shc to the EGF (23–25) and insulin (25, 26) receptors. Receptor-associated Shc is rapidly phosphorylated (27–29) and subsequently binds a Grb2·Sos complex, which results in the translocation of the complex to the plasma membrane (30, 31). Sos, a guanine nucleotide exchange protein, then activates Ras (32, 33), which in turn stimulates the mitogen-activated protein kinase (MAPK) cascade (34, 35). Shc has been implicated in mitogenic signaling by epidermal growth factor (36), platelet-derived

‡ Present address: Dept. of Cell Biology, Neurobiology and Anatomy, Loyola University Medical Center, Maywood, IL 60153.

§ To whom correspondence should be addressed. Tel.: 319-335-6508; Fax: 319-335-8930.

[1] The abbreviations used are: EGF, epidermal growth factor; Erk, extracellularly regulated kinase; DMEM, Dulbecco's modified Eagle's medium; Grb2, growth factor receptor-bound protein 2; GST, glutathione S-transferase; GST-B3, GST fusion protein incorporating rat ErbB3 residues 1311–1339; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; NPXY, Asn-Pro-Xaa-Tyr sequence motif; PAGE, polyacrylamide gel electrophoresis; PI, phosphoinositide; WT, wild-type; Y/F, Tyr → Phe amino acid substitution; YXXM, Tyr-Xaa-Xaa-Met sequence motif.

growth factor (37), nerve growth factor (38), and insulin (39) receptors.

The Shc protein has been shown to associate with phosphorylated ErbB3 (28, 40), and heregulin has been found to stimulate the phosphorylation of Shc (40). These findings suggest a possible contribution of the Shc signaling pathway to heregulin-stimulated mitogenesis. Synthetic phosphopeptide competition experiments have indicated that Tyr-1309 in human ErbB3 is the binding site of Shc (28). By mutating the corresponding tyrosine residue in the putative Shc binding site of the rat ErbB3 receptor protein, we have in the present study examined the heregulin-stimulated interaction of Shc with the ErbB3 receptor, and we have investigated the role of Shc in mitogenesis mediated by the ErbB2·ErbB3 coreceptor complex.

EXPERIMENTAL PROCEDURES

Materials—NIH-3T3 cells were purchased from American Type Culture Collection. LipofectAMINE transfection reagent was obtained from Life Technologies, Inc. Recombinant heregulin-β1 and antibodies recognizing ErbB2 (Ab-1) and ErbB3 (2C3, 2F12) were purchased from NeoMarkers. Anti-phosphotyrosine (PY20), recombinant PY20 conjugated to horseradish peroxidase, anti-Shc, and anti-Grb2 were purchased from Transduction Laboratories. Anti-p85 was purchased from Upstate Biotechnology. A mitogen-activated protein kinase-specific antibody recognizing both Erk1 and Erk2 isoforms (Zymed Laboratories Inc.) and distinct Erk1-specific and Erk2-specific antibodies (Santa Cruz) were also procured. Recombinant platelet-derived growth factor-BB and wortmannin were purchased from Sigma. Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (ECL) reagents were purchased from Amersham Pharmacia Biotech. [γ-^{32}P]ATP (3000 Ci/mmol) and [*methyl*-^{3}H]thymidine (90 Ci/mmol) were acquired from NEN Life Science Products. The recombinant EGF receptor protein tyrosine domain, consisting of amino acid residues 645–972 of the parent receptor, was expressed with the baculovirus/insect system and purified as described previously (5).

Generation of an ErbB3 Tyr-1325 → Phe Mutant Protein—The rat ErbB3 cDNA (16) was mutated by use of the Ex-Site Mutagenesis kit from Stratagene. A tyrosine codon corresponding to amino acid 1325 was replaced with a phenylalanine codon with a 33-base pair reverse mutagenic primer 5'-GGGAAAAGCCGGCTGTGCCAGAAATCGGGG-TTG-3' and the ErbB3 expression plasmid pcDNA3-B3 (16) as the template for the polymerase chain reaction mutagenesis. The altered region of the cDNA was subcloned into the parent expression vector to yield the mutant ErbB3 receptor cDNA expression vector (pcDNA3-B3-Y/F). The affected region was sequenced to verify the accuracy of polymerase chain reaction amplification.

Cell Culture—NIH-3T3 cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum at 37 °C in a 5% CO_2 atmosphere. After transfection with the pcDNA3-B3-Y/F mutant expression plasmid using LipofectAMINE reagent, stable NIH-3T3 clones were selected with Geneticin (G418) and screened for the expression of the mutant receptor protein by Western blotting. A stable NIH-3T3 cell line expressing ErbB3-WT was isolated as described previously (16). For some [^{3}H]thymidine incorporation assays nonclonal pools of NIH-3T3 cells transfected with pcDNA3-B3-WT and pcDNA3-B3-Y/F were grown under Geneticin selection. Equivalent expression of wild-type and mutant receptors was verified by immunoblotting.

Cell Stimulation, Immunoprecipitation, and Immunoblotting—Prior to stimulation with growth factor, cells were starved for 18 h in low serum medium (DMEM containing 0.1% fetal bovine serum). Starved cells were washed once with low serum medium and incubated with heregulin-β1 (1 nM final concentration) diluted in culture medium containing 0.1% bovine serum albumin, or the dilution vehicle, for 5–7 min at 37 °C. Cells were washed immediately with ice-cold phosphate-buffered saline and lysed with Nonidet P-40 lysis buffer (1% Nonidet P-40, 50 mM Hepes/Na, 150 mM sodium chloride, 2 mM EDTA, 3 mM EGTA, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 2 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, pH 7.4). The whole cell lysates were centrifuged for 10 min at 13,000 × g. After protein concentration was assayed, the supernatants were immunoprecipitated with appropriate antibodies (8). The immunoprecipitates and cell lysate samples were resolved by SDS-PAGE, transferred to a polyvinylidene

difluoride membrane, and detected with the indicated antibodies by ECL luminography.

In Vitro Binding Assays—A C-terminal peptide fragment of ErbB3 (residues 1311–1339) containing Tyr-1325 was generated as a GST fusion protein (GST-B3) and purified as described previously (41). GST-B3 or GST (65 pmol each) was incubated in buffer A (20 mM Hepes/Na, 50 mM sodium chloride, 10% (v/v) glycerol, pH 7.4) supplemented with 10 mM $MnCl_2$, 0.1% Triton X-100, and 0.1 μg of EGF receptor protein tyrosine kinase domain (5) in the absence or presence of 50 μM ATP for 30 min at 22 °C (total volume 10 μl). The mixtures were diluted into 375 μl of lysate from NIH-3T3 cells (2 mg/ml protein), incubated for 30 min on ice, and then allowed to bind glutathione-agarose (100 μl of a 1:1 suspension in buffer A) for 1 h at 4 °C. The agarose suspensions were centrifuged for 1 min at 600 × g. The pellets were washed twice in 500 μl of ice-cold Nonidet P-40 lysis buffer and then suspended in gel sample buffer. Pellets and cell lysate samples (20 μg of protein) were resolved by SDS-PAGE and immunoblotted with anti-Shc and anti-phosphotyrosine.

Mitogen-activated Protein Kinase Assay—Mitogen-activated protein kinase (MAPK) from cells stimulated with heregulin or control vehicle was immunoprecipitated with a combination of Erk1 and Erk2 antibodies as described above. The washed immunoprecipitates were suspended in 30 μl of reaction buffer containing 10 mM Hepes/Na, 10 mM $MgCl_2$, pH 7.4, and 8 μg of myelin basic protein (MBP). The reaction was initiated by adding 3 μl of 100 μM ATP containing 5 μCi of [γ-^{32}P]ATP and incubated for 15 min at 30 °C. The reaction was quenched with sample buffer, and the proteins were subjected to SDS-PAGE. The gel was subsequently dried, exposed to autoradiographic film, and MBP phosphorylation quantified by scintillation counting of excised gel bands. In the MAPK gel shift assay, cell lysate supernatants from heregulin-, platelet-derived growth factor-, or vehicle-stimulated cells were subjected to Western blotting with an antibody recognizing both the Erk1 and Erk2 isoforms of MAPK. Here in SDS-PAGE the amount of bisacrylamide in the gel was reduced (acrylamide:bisacrylamide, 30:0.04), and the electrode buffer was twice-concentrated (42).

[^{3}H]Thymidine Incorporation Assay—Cells were plated at a density of 5 × 10^5/well in 6-well dishes, grown for 24 h, and then serum-deprived for 18 h in DMEM containing 0.1% fetal bovine serum. Cells were then stimulated with varying concentrations of heregulin-β1 for 18 h, after which 0.5 μCi/ml of [*methyl*-^{3}H]thymidine was added to each well, and the cells were further incubated for 4 h. For experiments with wortmannin, either Me_2SO or wortmannin (100 nM) in Me_2SO was added 30 min prior to stimulation of cells with either vehicle or heregulin (10 nM). Cells were then washed twice with cold phosphate-buffered saline, extracted with 5% trichloroacetic acid, and then solubilized in 0.1 M sodium hydroxide. The radioactivity incorporated into DNA was measured by scintillation counting.

RESULTS

Heregulin-dependent Phosphorylation of Wild-type and Mutant ErbB3 Proteins in Stably Transfected NIH-3T3 Cells—By using site-directed mutagenesis, we created a mutant ErbB3 protein in which the candidate Shc binding site residue, Tyr-1325 (28), was substituted with phenylalanine (ErbB3-Y/F). NIH-3T3 fibroblast cell lines that stably expressed high levels of the wild-type (ErbB3-WT) and mutant (ErbB3-Y/F) receptor proteins were isolated. To confirm the expression of the receptor proteins, cell lysates were analyzed by immunoprecipitation followed by Western blotting with an ErbB3-specific antibody. The transfected fibroblasts expressed comparable levels of wild-type and mutant ErbB3 proteins (Fig. 1A). Cells transfected with the parent expression vector did not express detectable ErbB3.

As we have previously observed (16), ErbB3-WT showed a constitutive phosphorylation on tyrosine residues that was enhanced by stimulation with heregulin-β1 (Fig. 1A). Phosphorylation of the ErbB3-Y/F mutant protein was enhanced to a similar extent as the wild-type protein. Treatment of the mock-transfected cells with heregulin induced no phosphorylation response. The heregulin-dependent phosphorylation of tyrosine residues in ErbB3 was presumably mediated by the ErbB2 protein tyrosine kinase, endogenously present in the NIH-3T3 fibroblasts (Fig. 1B). The endogenous ErbB2 protein in NIH-3T3 cells expressing either ErbB3-WT or ErbB3-Y/F was phos-
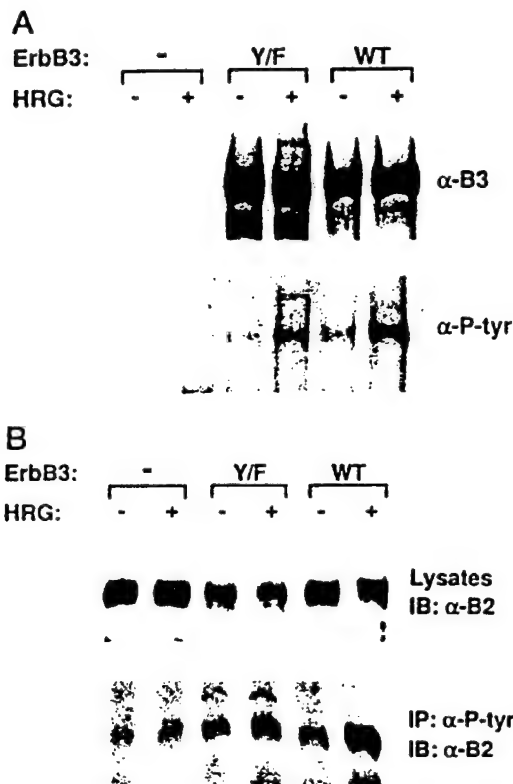
A

ErbB3: - Y/F WT
HRG: - + - + - +

α-B3

α-P-tyr

B

ErbB3: - Y/F WT
HRG: - + - + - +

Lysates
IB: α-B2

IP: α-P-tyr
IB: α-B2

FIG. 1. **Expression and heregulin-mediated phosphorylation of ErbB2 and ErbB3 in stably transfected NIH-3T3 cell lines.** NIH-3T3 cells were transfected with either the parent pcDNA3 expression vector (−) or pcDNA3 incorporating wild-type (*WT*) and Tyr-1325 → Phe mutant (*Y/F*) ErbB3 cDNAs. Mock-transfected cells and cells expressing ErbB3-Y/F and ErbB3-WT were serum-starved and stimulated with vehicle or 1 nM heregulin-β1. *A*, cell lysates containing 1 mg of protein were immunoprecipitated with ErbB3-specific antibody, and the immunoprecipitates were subjected to SDS-PAGE and immunoblotting with either ErbB3-specific antibody (*α-B3*) or anti-phosphotyrosine antibody (*α-P-tyr*). *B*, alternatively, cell lysates were blotted with ErbB2-specific antibody (*α-B2*) or immunoprecipitated (*IP*) with a phosphotyrosine-specific antibody (*α-P-Tyr*) and then immunoblotted (*IB*) with ErbB2-specific antibody.

phorylated on tyrosine residues, and this phosphorylation was augmented in response to heregulin (Fig. 1*B*), which was consistent with the observation that ErbB2 and ErbB3 function as heregulin coreceptors (10, 43).

The Tyr-1325 → Phe Point Mutation in ErbB3 Abolishes Heregulin-dependent ErbB3/Shc Association—To assay the association of Shc with the wild-type and mutant ErbB3 receptor proteins, lysates of stably transfected NIH-3T3 cells were immunoprecipitated with an Shc-specific antibody and subsequently immunoblotted with an ErbB3-specific antibody. Lysates from mock-transfected cells and cells expressing either the mutant or wild-type receptor protein showed the presence of each isoform of Shc, p46, p52, and p66, in similar amounts across the cell lines (Fig. 2*A*). From cells expressing the wild-type receptor, the ErbB3 protein coimmunoprecipitated with Shc, which suggested that Shc constitutively associated with ErbB3. However, this ErbB3/Shc association was significantly enhanced following stimulation with heregulin. In contrast, Shc immunoprecipitates from cells expressing ErbB3-Y/F showed no presence of the ErbB3-Y/F protein (Fig. 2*B*). Thus, the mutation of a single tyrosine in the NPXY sequence motif in the ErbB3 receptor abolished association of Shc with ErbB3. Mock-transfected cells showed no heregulin-dependent ErbB3/Shc association. Interestingly, no association of Shc with ErbB2 was evident in any of the cells (Fig. 2*B*) (see "Discussion").

Heregulin-stimulated Shc Phosphorylation and Shc/Grb2 Association Is Significantly Attenuated in NIH-3T3 Cells Expressing ErbB3-Y/F—Since the Tyr → Phe mutation blocked the interaction of Shc with the ErbB3 receptor, we examined the effect of this mutation on heregulin signaling by the ErbB2·ErbB3 coreceptor. To investigate potential heregulin-stimulated Shc phosphorylation, Shc immunoprecipitates were probed with a phosphotyrosine-specific antibody. An increase in the phosphorylation of the Shc proteins was seen in response to heregulin in cells expressing the wild-type receptor. Among the three isoforms of Shc, p52 seemed to be preferentially phosphorylated in response to heregulin. In cells expressing ErbB3-Y/F the heregulin-induced phosphorylation of Shc was significantly reduced as compared with cells expressing the wild-type receptor (Fig. 2*C*). Heregulin did not stimulate Shc phosphorylation in the mock-transfected cells.

Anti-Shc immunoprecipitates were also probed with a Grb2-specific antibody. Grb2 was found to be constitutively associated with Shc, but this association was increased in response to heregulin in cells expressing ErbB3-WT. No increase in heregulin-stimulated Shc/Grb2 association was seen in cells expressing the ErbB2-Y/F mutant receptor. Vector-transfected cells also showed no heregulin-dependent Shc/Grb2 association (Fig. 2*C*). These results indicated that Shc phosphorylation and Shc/Grb2 association were potentiated by the binding of Shc to ErbB3, which was apparently mediated by Tyr-1325 in the ErbB3 C terminus.

A Phosphorylated ErbB3 C-terminal Peptide Interacts with Shc Proteins in Vitro—To determine whether Tyr-1325 in the ErbB3 C terminus could when phosphorylated serve as a binding site for the Shc protein, we expressed a short C-terminal peptide fragment of ErbB3 (residues 1311–1339) containing only one tyrosine residue, Tyr-1325, as a GST fusion protein (GST-B3), and we used this protein in *in vitro* binding assays. Here the GST-B3 fusion protein was first phosphorylated with a recombinant EGF receptor protein tyrosine kinase domain (5) and then incubated with lysates of NIH-3T3 cells containing the Shc proteins. After precipitation of the phosphorylated GST-B3 protein with glutathione-agarose, associated Shc proteins were detected by Western blotting (Fig. 2*D*). Control experiments showed that the GST domain was not phosphorylated under these conditions and did not significantly interact with the Shc proteins. Also, the interaction of Shc with GST-B3 was dependent upon prior phosphorylation of the fusion protein. These results indicated that the C-terminal NPXY motif in ErbB3 could serve when phosphorylated as an Shc-binding site.

Heregulin-stimulated Activation of MAPK Is Impaired in NIH-3T3 Cells Expressing the ErbB3-Y/F Mutant Protein—Receptor-mediated Shc phosphorylation and Shc/Grb2 association would be predicted to result in the activation of the Ras/MAPK signaling pathway. Potential heregulin-stimulated activation of MAPK was characterized in NIH-3T3 cells expressing ErbB2·ErbB3 coreceptors (Fig. 3). The ErbB3-Y/F receptor protein, which failed to interact with Shc, was used to examine the involvement of Shc in the activation of MAPK via the ErbB2·ErbB3 coreceptor. The activation of MAPK in the NIH-3T3 transfectants was detected by gel mobility shift assays (Fig. 3*A*) and *in vitro* phosphorylation assays employing the exogenous substrate myelin basic protein (MBP) (Fig. 3*B*). In NIH-3T3 cells expressing ErbB3-WT, MAPK was clearly activated in response to heregulin. This was evident by the retarded migration of both the p42 (Erk2) and p44 (Erk1) isoforms of MAPK in gel shift assays (Fig. 3*A*). Also, MAPK immunoprecipitates from heregulin-stimulated cells expressing ErbB3-WT showed strong MBP phosphorylation in immune
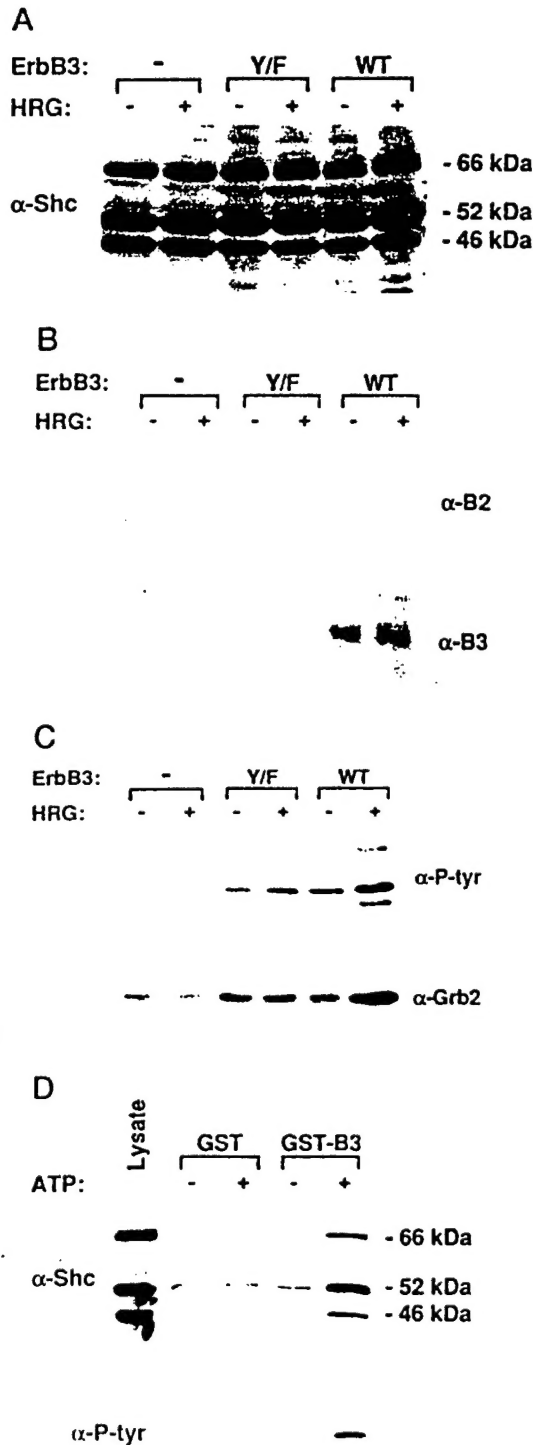
A

ErbB3: -   Y/F   WT
HRG: - +   - +   - +

α-Shc

- 66 kDa
- 52 kDa
- 46 kDa

B

ErbB3: -   Y/F   WT
HRG: - +   - +   - +

α-B2

α-B3

C

ErbB3: -   Y/F   WT
HRG: - +   - +   - +

α-P-tyr

α-Grb2

D

ATP: GST GST-B3
       - +   - +

α-Shc

- 66 kDa
- 52 kDa
- 46 kDa

α-P-tyr

FIG. 2. **Heregulin-stimulated ErbB3/Shc association, Shc phosphorylation, and Shc/Grb2 association.** NIH-3T3 cells transfected with vector, ErbB3-WT, or ErbB3-Y/F cDNAs were treated as described in Fig. 1. *A*, lysates from cells stimulated with heregulin or control vehicle were probed with an Shc-specific antibody (*α-Shc*). All three isoforms of Shc (p46, p52, and p66) were evident. *B*, Shc was immunoprecipitated from the lysates with a Shc-specific antibody, and the immunoprecipitates were immunoblotted with either ErbB2-specific (*α-B2*) or ErbB3-specific (*α-B3*) antibody. *C*, Shc immunoprecipitates were also immunoblotted with an anti-phosphotyrosine-horseradish peroxidase conjugate (*α-P-tyr*) or a Grb2-specific antibody (*α-Grb2*). *D*, *in vitro* association of the phosphorylated ErbB3 C terminus with Shc proteins. GST-B3 and GST (65 pmol each) were incubated under phosphorylating (+ATP) or nonphosphorylating (−ATP) conditions, allowed to interact with Shc proteins from NIH-3T3 cell lysates, and then precipitated with glutathione-agarose. Association of Shc proteins with precipitated GST-B3 or GST was detected by immunoblotting with a
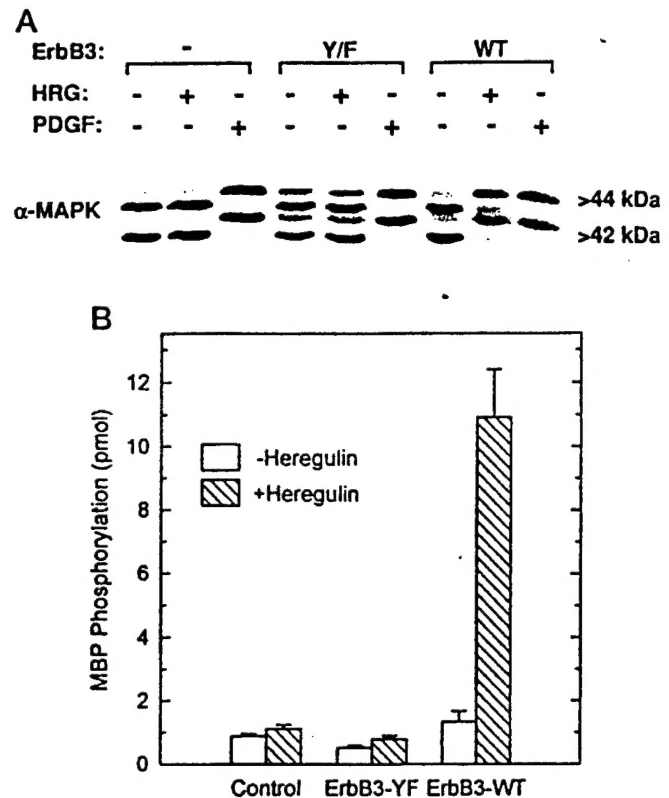
A

ErbB3: -   Y/F   WT
HRG: - + -   - + -   - + -
PDGF: - - +   - - +   - - +

α-MAPK

>44 kDa
>42 kDa

B

FIG. 3. **Heregulin-stimulated activation of mitogen-activated protein kinase.** *A*, cells stimulated with heregulin (1 nM), platelet-derived growth factor (50 ng/ml), or vehicle were lysed as described in Fig. 1. Cell lysates containing 70 µg of total protein were subjected to a gel mobility shift assay of MAPK activation as described under "Experimental Procedures." Both the p42 and p44 isoforms of MAPK are indicated. The appearance of more slowly migrating bands in cells transfected with wild-type ErbB3 cDNA in response to heregulin and in all three cell lines in response to platelet-derived growth factor indicated the activation of the MAPK isoforms. *B*, alternatively, MAPK was immunoprecipitated from heregulin-stimulated and control cells, and the immunoprecipitates were subjected to *in vitro* MAPK assays with MBP and [γ-³²P]ATP as substrates. *Error bars* represent the standard error of three independent experiments.

complex kinase assays (Fig. 3*B*). In contrast, NIH-3T3 cells expressing ErbB3-Y/F showed no MAPK activation in response to heregulin. Since NIH-3T3 fibroblasts endogenously express receptors for platelet-derived growth factor, it was of interest to see whether this factor stimulated the activation of MAPK in the various transfected cell lines. MAPK was clearly activated in response to platelet-derived growth factor in the mock-transfected cells and in cells expressing either ErbB3-Y/F or ErbB3-WT, as was evident by the retarded migration of both the p42 and p44 isoforms of MAPK in the gel shift assay (Fig. 3*A*). Possibly, the failure of the ErbB3-Y/F protein to activate MAPK in response to heregulin resulted from its inability to interact with Shc and mediate an Shc/Grb2 association.

Association of PI 3-Kinase with Wild-type and Mutant ErbB3 Proteins in Transfected NIH-3T3 Fibroblasts—Previous studies have reported an association of PI 3-kinase with the ErbB3 protein in both the EGF receptor-ErbB3 (8, 9) and ErbB2-ErbB3 (11) coreceptor contexts. Given that ErbB3-Y/F failed to both interact with Shc and activate MAPK, we sought to determine if the mutant receptor could still associate with PI

Shc-specific antibody (*α-Shc*). An aliquot of the original cell lysates corresponding to ~1/40 of that in the binding assay was also analyzed and shown for comparison. Phosphorylation of the ErbB3 C-terminal peptide was detected by immunoblotting with anti-phosphotyrosine (*α-P-tyr*).
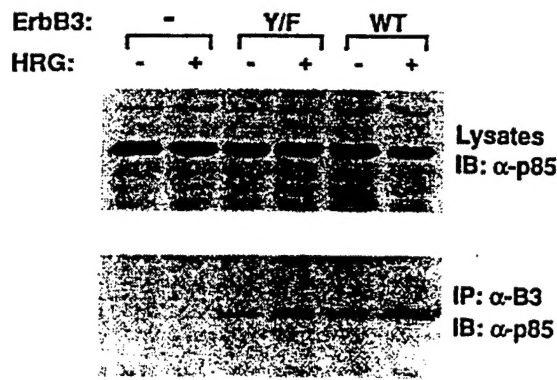
ErbB3:   -   Y/F   WT
HRG: -   +   -   +   -   +

Lysates
IB: α-p85

IP: α-B3
IB: α-p85

FIG. 4. **Association of PI 3-kinase with ErbB3-WT and ErbB3-Y/F in stably transfected NIH-3T3 fibroblasts.** Transfected cells were treated as described in Fig. 1. Lysates from vehicle- or heregulin-stimulated cells were probed with an antibody recognizing the p85 regulatory subunit of PI 3-kinase (α-p85). Cell lysates containing 1 mg of protein were also immunoprecipitated (*IP*) with ErbB3-specific antibody (α-B3), and the immunoprecipitates were subjected to SDS-PAGE and immunoblotting (*IB*) with p85-specific antibody.

3-kinase and therefore potentially signal through the PI 3-kinase pathway. Immunoblotting analyses of ErbB3 immunoprecipitates from control cells and cells expressing either ErbB3-WT or ErbB3-Y/F showed the presence of the p85 regulatory subunit of PI 3-kinase (Fig. 4), which indicated that the mutant ErbB3 protein retained its ability to interact with PI 3-kinase. Heregulin-stimulated association of p85 with ErbB3 was variable, which could have been due to the high basal association seen in the transfected NIH-3T3 cells.

Heregulin-stimulated DNA Synthesis in NIH-3T3 Cells Expressing ErbB3-WT and ErbB3-Y/F—In order to determine whether the wild-type and mutant ErbB3 proteins mediated a mitogenic response to heregulin, cellular DNA synthesis was analyzed with a [3H]thymidine incorporation assay. The results of a representative experiment are shown in Fig. 5A. Mock-transfected NIH-3T3 cells showed no enhanced [3H]thymidine uptake in response to heregulin. Cells expressing ErbB3-WT showed a dose-dependent uptake of [3H]thymidine with a significant stimulation seen at a 0.1 nM concentration of heregulin. Cells expressing ErbB3-Y/F showed an attenuated mitogenic response relative to those expressing the wild-type receptor protein. Interestingly, the high basal activity seen in the cells expressing ErbB3-WT was absent in cells expressing ErbB3-Y/F. Heregulin-stimulated [3H]thymidine incorporation, defined as the difference between basal incorporation and that stimulated by 10 nM heregulin, was compared between cells expressing either ErbB3-Y/F or ErbB3-WT (see Fig. 6). In five separate experiments, heregulin-stimulated DNA synthesis mediated by ErbB3-Y/F was found to range between 15 and 60% that mediated by ErbB3-WT. Heregulin-stimulated DNA synthesis was also studied with nonclonal pools of cells transfected with ErbB3-WT and ErbB3-Y/F cDNAs to ensure that the attenuated mitogenic response seen with clonal cells expressing ErbB3-Y/F was not an effect of clonal variation. Fig. 5B shows the results of a representative experiment with nonclonal cells expressing moderate levels of the wild-type and mutant receptor. Nonclonal cells expressing ErbB3-Y/F showed a significantly attenuated mitogenic response when compared with cells expressing ErbB3-WT. These results indicated that the association of Shc with the ErbB3 protein and the ensuing activation of the Ras/MAPK signaling pathway contributed to the mitogenic potential of the ErbB2·ErbB3 heregulin coreceptor.

Effect of Wortmannin on Heregulin-stimulated [3H]Thymidine Incorporation—Given that the ErbB3-Y/F protein re-
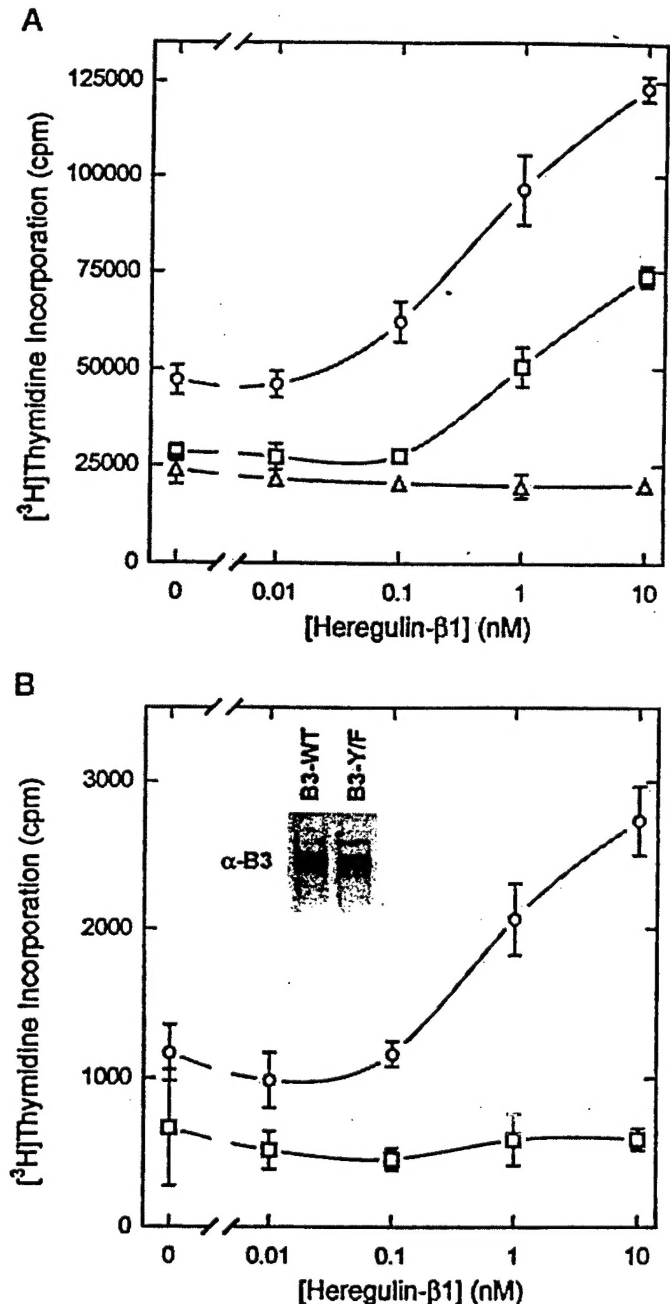
A

B

FIG. 5. **Heregulin-stimulated [3H]thymidine uptake by NIH-3T3 cells expressing ErbB3-WT and ErbB3-Y/F.** *A*, NIH-3T3 clones stably transfected with pcDNA3 (△), pcDNA3-B3-WT (○), or pcDNA3-B3-Y/F (□) were serum-starved overnight followed by treatment with varying concentrations of heregulin for 18 h. *methyl-*[3H]thymidine was added to the stimulation medium, and its incorporation into DNA was determined after 4 h. *B*, NIH-3T3 cells transfected with pcDNA3-B3-WT (○), or pcDNA3-B3-Y/F (□) were grown under Geneticin selection for 4 weeks to generate nonclonal pools expressing wild-type and mutant receptors. [3H]Thymidine incorporation was analyzed as in *A*. The *inset* shows expression levels of ErbB3-WT and ErbB3-Y/F in the nonclonal transfected cells as determined by immunoblotting of cell lysates with ErbB3 antibody (α-B3). *Error bars* represent the standard deviation of triplicate assays.

tained the ability to associate with the p85 regulatory subunit of PI 3-kinase (Fig. 4), it was considered that activation of the PI 3-kinase pathway might account for the residual mitogenic activity seen in cells expressing ErbB3-Y/F. To determine the contribution of PI 3-kinase to the stimulation of DNA synthesis by heregulin, we examined the effect of wortmannin, a PI 3-kinase inhibitor, on [3H]thymidine uptake in cells expressing either ErbB3-WT or ErbB3-Y/F (Fig. 6). Cells were treated with
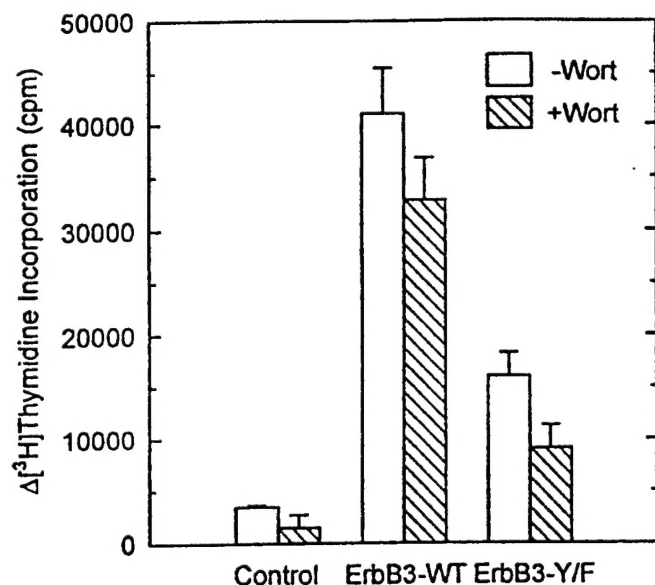
FIG. 6. **Effect of inhibition of PI 3-kinase activity on heregulin-stimulated [³H]thymidine uptake.** Serum-starved cells transfected with pcDNA3, pcDNA3-B3-Y/F, or pcDNA3-B3-WT expression vectors were incubated with wortmannin (*Wort*; 100 nM) or vehicle for 30 min prior to stimulation with either vehicle or heregulin (10 nM) for 18 h. [³H]Thymidine incorporation assays were performed as indicated in Fig. 5. Heregulin-stimulated [³H]thymidine incorporation (Δ[³H]thymidine incorporation) was determined in triplicate assays by subtracting basal incorporation from that stimulated by 10 nM heregulin.

or without wortmannin for 30 min prior to stimulation with either vehicle or 10 nM heregulin. In the representative experiment shown in Fig. 6, heregulin-stimulated DNA synthesis mediated by ErbB3-Y/F was found to be 39% that mediated by ErbB3-WT. Wortmannin decreased heregulin-stimulated [³H]thymidine incorporation in cells expressing ErbB3-Y/F by almost 45% and to a lesser extent (20%) in cells expressing ErbB3-WT. These results implicated PI 3-kinase as another contributor in mitogenic signaling by ErbB2·ErbB3 heregulin coreceptors. A similar effect of wortmannin on heregulin-stimulated DNA synthesis was previously observed in a study of fibroblasts expressing ectopic ErbB2 and ErbB3 proteins (11).

DISCUSSION

The ErbB2 and ErbB3 proteins together constitute a functional heregulin coreceptor (10). Whereas heregulin is a ligand for the ErbB3 receptor protein, ErbB2 does not independently bind heregulin with significant affinity (10, 43), although it may in the context of an ErbB2·ErbB3 heterodimer cooperate in the high affinity binding of heregulin (10). The ErbB3 protein appears to be devoid of intrinsic kinase activity (4, 5). However, C-terminal tyrosine residues of both ErbB2 and ErbB3 are phosphorylated upon stimulation of the ErbB2·ErbB3 coreceptor (10), which is apparently mediated by the protein tyrosine kinase activity of ErbB2 (12). Hence, both ErbB2 and ErbB3, by complementing the functions of one another, can play important roles in heregulin signaling.

Heterodimerization might also increase the diversity of signaling through the activated ErbB2·ErbB3 coreceptor. However, the signal transduction pathways activated by the ErbB2·ErbB3 coreceptor have not been thoroughly characterized. The coupling of PI 3-kinase to ErbB3 in response to heregulin in the ErbB2·ErbB3 coreceptor context (11) and in response to EGF in cells overexpressing EGF receptor and ErbB3 (8, 9) has been documented. The Shc adapter protein has been shown to be phosphorylated in response to heregulin in cells overexpressing both ErbB3 and ErbB4 (40) and in cells overexpressing ErbB4 alone (44). The identification of the po-

tential binding site of Shc on the ErbB3 C terminus by use of peptide competition assays (28) and the heregulin-stimulated ErbB3/Shc association demonstrated in cells expressing the ErbB2 and ErbB3 proteins (7, 40) have implicated Shc in heregulin signal transduction.

The purposes of this study were to demonstrate the binding of Shc to a specific residue in the ErbB3 C terminus in response to heregulin and to determine if this heregulin-induced binding event contributed to the mitogenic response elicited by the ErbB2·ErbB3 coreceptor. We addressed these questions by site-directed mutagenesis of Tyr-1325 in the putative Shc binding site (NPXY motif) (21–24) in the ErbB3 C terminus. Expression of the ErbB3-Y/F mutant protein in NIH-3T3 fibroblasts expressing endogenous ErbB2 resulted in the formation of functional heregulin coreceptors (Fig. 1). Heregulin stimulated the phosphorylation of the mutant ErbB3 protein to a similar extent as the wild-type protein (Fig. 1A). However, the Tyr-1325 → Phe substitution abolished interaction of ErbB3 with Shc (Fig. 2B), which suggested that Shc specifically bound to phosphorylated Tyr-1325 in the ErbB3 C terminus. The potential of phosphorylated Tyr-1325 of ErbB3 to interact with Shc proteins was subsequently demonstrated by *in vitro* binding experiments (Fig. 2D). The observations that heregulin did not (i) stimulate the phosphorylation of Shc (Fig. 2C), (ii) stimulate association of Shc with Grb2 (Fig. 2C), or (iii) activate MAPK (Fig. 3) in NIH-3T3 cells expressing the ErbB3-Y/F receptor suggested that heregulin-stimulated ErbB3/Shc association was necessary for the activation of these downstream signaling events. Also, it was apparent that any possible interaction of Grb2 with the activated ErbB2 or ErbB3 protein could not effectively activate the Ras/MAPK signaling pathway in the absence of Shc involvement.

Previous studies of NIH-3T3 fibroblasts (29) and T47D mammary carcinoma cells (40) have reported an ErbB2/Shc interaction. In the former study, a chimeric EGF receptor/ErbB2 protein was expressed in NIH-3T3 fibroblasts, and an EGF-dependent association of Shc with the ErbB2 cytoplasmic domain was seen. The latter study of T47D cells documented a heregulin-stimulated ErbB2/Shc association in addition to ErbB3/Shc association. Also, the catalytically activated rat *ErbB2/Neu* oncogene product was found to interact with Shc via an Asn-Leu-Tyr-Tyr (NLYY) sequence motif in the receptor C terminus (20, 45). In contrast, we failed to see any ErbB2/Shc interaction in the NIH-3T3 transfectants in response to heregulin (Fig. 2B). One possible explanation for these apparent discrepancies is that in each of these cases phosphorylation of the ErbB2 cytosolic domain occurred in the context of a coreceptor complex with different constituents, which could have resulted in the phosphorylation of distinct subsets of tyrosine residues in the ErbB2 C terminus. In the cases of the chimeric EGF receptor/ErbB2 protein and the *ErbB2/Neu* oncogene product, ErbB2 phosphorylation presumably was mediated by the ErbB2 catalytic domain. In the case of T47D cells, which express all four members of the ErbB family, the ErbB2 protein may have been phosphorylated within a heterodimeric complex with the kinase-active heregulin receptor ErbB4. In the present case, ErbB2 phosphorylation likely occurred in the context of a dimeric complex with the kinase-deficient ErbB3 protein. ErbB2 phosphorylation in this context would require either an intramolecular mechanism (46) or a mechanism involving higher order receptor oligomers (47, 48). Alternatively, our failure to detect ErbB2/Shc association in NIH-3T3 cells overexpressing recombinant ErbB3 in the presence of endogenous ErbB2 might have reflected a relatively low ratio of ErbB2 and ErbB3 protein levels. Indeed, Pinkas-Kramarski *et al.* (7) have

previously demonstrated an ErbB2/Shc association in cells overexpressing both ErbB2 and ErbB3.

Heregulin is mitogenic to a variety of cell types (49) including human mammary cancer cells (2) in which ErbB3 and other members of the ErbB family are often overexpressed. The ErbB2·ErbB3 heterodimeric complex has been shown to be capable of mediating mitogenic and proliferative responses to heregulin, and PI 3-kinase has been shown to be involved in these responses (11). Because the binding of Shc to the ErbB2·ErbB3 coreceptor expressed in our transfected fibroblast cell lines appeared to be directly mediated by the phosphorylation of Tyr-1325 of ErbB3, the Tyr-1325 → Phe mutant ErbB3 protein could be exploited in the investigation of the role of Shc in mitogenic signaling by the ErbB2·ErbB3 heregulin coreceptor.

Whereas heregulin stimulated a dose-dependent increase in DNA synthesis in cells expressing ErbB3-WT, this response was significantly attenuated in cells expressing ErbB3-Y/F (Fig. 5). The high basal mitogenic activity displayed by cells expressing ErbB3-WT was not shown by cells expressing ErbB3-Y/F. Qualitatively similar results were observed when either clonal cells expressing high levels of ErbB3-WT and ErbB3-Y/F or nonclonal pools of cells expressing moderate levels of the ErbB3 proteins were examined, although the residual mitogenic activity of the ErbB3-Y/F protein was enhanced in the clonal cells. The heregulin-stimulated component of DNA synthesis (Δ[^{3}H]thymidine incorporation) in clonal cells expressing ErbB3-Y/F was found to be significantly lower than in clonal cells expressing ErbB3-WT (Fig. 6). The residual mitogenic response to heregulin seen in the cells expressing ErbB3-Y/F could have reflected the activation of the PI 3-kinase pathway (11), which would presumably not be blocked by the Shc binding site mutation. Indeed, ErbB3-Y/F was able to associate with the p85 regulatory subunit of PI 3-kinase to a similar extent as was ErbB3-WT (Fig. 4). Also, pretreatment with the PI 3-kinase inhibitor wortmannin decreased heregulin-stimulated [^{3}H]thymidine uptake in cells expressing ErbB3-Y/F as well as in cells expressing ErbB3-WT (Fig. 6). Whereas the residual mitogenic activity seen in cells expressing ErbB3-Y/F might therefore be attributed in part to the activation of the PI 3-kinase pathway, we conclude that Shc-mediated signaling events contributed significantly to mitogenic signaling by the ErbB2·ErbB3 heregulin coreceptor.

In summary, the results presented in this study indicated that Tyr-1325 in the ErbB3 C terminus is a primary site for the interaction of Shc with the ErbB2·ErbB3 coreceptor complex. Mutation of this tyrosine to phenylalanine abolished association of Shc with ErbB3, blocked activation of the MAPK signaling pathway, and attenuated the mitogenic response to heregulin. Our studies have thus demonstrated that heregulin-induced association of Shc with ErbB3 can initiate signaling events that contribute significantly to the mitogenic effect of heregulin on cells expressing ErbB2·ErbB3 coreceptors.

REFERENCES

1. Fantl, W. J., Johnson, D. E., and Williams, L. T. (1993) *Annu. Rev. Biochem.* **62**, 453–481
2. Holmes, W. E., Sliwkowski, M. X., Akita, R. W., Henzel, W. J., Lee, J., Park, J. W., Yansura, D., Abadi, N., Raab, H., Lewis, G. D., Shepard, H. M., Kuang, W. J., Wood, W. I., Goeddel, D. V., and Vandlen, R. L. (1992) *Science* **256**, 1205–1210
3. Wen, D., Peles, E., Cupples, R., Suggs, S. V., Bacus, S. S., Luo, Y., Trail, G., Hu, S., Silbiger, S. M., Levy, R. B., Koski, R. A., Lu, H. S., and Yarden, Y. (1992) *Cell* **69**, 559–572
4. Guy, P. M., Platko, J. V., Cantley, L. C., Cerione, R. A., and Carraway, K. L., III (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 8132–8136
5. Sierke, S. L., Cheng, K., Kim, H.-H., and Koland, J. G. (1997) *Biochem. J.* **322**, 757–763
6. Carraway, K. L., III, and Cantley, L. C. (1994) *Cell* **78**, 5–8
7. Pinkas-Kramarski, R., Soussan, L., Waterman, H., Levkowitz, G., Alroy, I., Klapper, L., Lavi, S., Seger, R., Ratzkin, B. J., Sela, M., and Yarden, Y. (1996) *EMBO J.* **15**, 2452–2467
8. Kim, H.-H., Sierke, S. L., and Koland, J. G. (1994) *J. Biol. Chem.* **269**, 24747–24755
9. Soltoff, S. P., Carraway, K. L., III, Prigent, S. A., Gullick, W. G., and Cantley, L. C. (1994) *Mol. Cell. Biol.* **14**, 3550–3558
10. Sliwkowski, M. X., Schaefer, G., Akita, R. W., Lofgren, J. A., Fitzpatrick, V. D., Nuijens, A., Fendly, B. M., Cerione, R. A., Vandlen, R. L., and Carraway, K. L., III (1994) *J. Biol. Chem.* **269**, 14661–14665
11. Carraway, K. L., III, Soltoff, S. P., Diamonti, A. J., and Cantley, L. C. (1995) *J. Biol. Chem.* **270**, 7111–7116
12. Kim, H.-H., Vijapurkar, U., Hellyer, N. J., Bravo, D., and Koland, J. G. (1998) *Biochem. J.* **334**, 189–195
13. Plowman, G. D., Green, J. M., Culouscou, J. M., Carlton, G. W., Rothwell, V. M., and Buckley, S. (1993) *Nature* **366**, 473–475
14. Tzahar, E., Levkowitz, G., Karunagaran, D., Yi, L., Peles, E., Lavi, S., Chang, D., Liu, N., Yayon, A., Wen, D., and Yarden, Y. (1994) *J. Biol. Chem.* **269**, 25226–25233
15. Pinkas-Kramarski, R., Shelly, M., Glathe, S., Ratzkin, B. J., and Yarden, Y. (1996) *J. Biol. Chem.* **271**, 19029–19032
16. Hellyer, N. J., Kim, H.-H., Greaves, C. H., Sierke, S. L., and Koland, J. G. (1995) *Gene (Amst.)* **165**, 279–284
17. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B., and Cantley, L. C. (1993) *Cell* **72**, 767–778
18. Cantley, L. C., Auger, K. R., Carpenter, C., Duckworth, B., Graziani, A., Kapeller, R., and Soltoff, S. (1991) *Cell* **64**, 281–302
19. Laminet, A. A., Apell, G., Conroy, L., and Kavanaugh, W. M. (1996) *J. Biol. Chem.* **271**, 264–269
20. Kavanaugh, W. M., Turck, C. W., and Williams, L. T. (1995) *Science* **268**, 1177–1179
21. Blaikie, P., Immanuel, D., Wu, J., Li, N., Yajnik, V., and Margolis, B. (1994) *J. Biol. Chem.* **269**, 32031–32034
22. Zhou, M.-M., Harlan, J. E., Wade, W. S., Crosby, S., Ravichandran, K. S., Burakoff, S. J., and Fesik, S. W. (1995) *J. Biol. Chem.* **270**, 31119–31123
23. Batzer, A. G., Blaikie, P., Nelson, K., Schlessinger, J., and Margolis, B. (1995) *Mol. Cell. Biol.* **15**, 4403–4409
24. Okabayashi, Y., Kido, Y., Okutani, T., Sugimoto, Y., Sakaguchi, K., and Kasuga, M. (1994) *J. Biol. Chem.* **269**, 18674–18678
25. Ricketts, W. A., Rose, D. W., Shoelson, S., and Olefsky, J. M. (1996) *J. Biol. Chem.* **271**, 26165–26169
26. Gustafson, T. A., He, W., Craparo, A., Schaub, C. D., and O'Neill, T. J. (1995) *Mol. Cell. Biol.* **15**, 2500–2508
27. Batzer, A. G., Rotin, D., Ureña, J. M., Skolnik, E. Y., and Schlessinger, J. (1994) *Mol. Cell. Biol.* **14**, 5192–5201
28. Prigent, S. A., and Gullick, W. J. (1994) *EMBO J.* **13**, 2831–2841
29. Segatto, O., Pelicci, G., Giuli, S., Digiesi, G., Di Fiore, P. P., McGlade, J., Pawson, T., and Pelicci, P. G. (1993) *Oncogene* **8**, 2105–2112
30. Rozakis-Adcock, M., McGlade, J., Mbamalu, G., Pelicci, G., Daly, R., Li, W., Batzer, A., Thomas, S., Brugge, J., Pelicci, P. G., Schlessinger, J., and Pawson, T. (1992) *Nature* **360**, 689–692
31. Rozakis-Adcock, M., Fernley, R., Wade, J., Pawson, T., and Bowtell, D. (1993) *Nature* **363**, 83–85
32. Chardin, P., Camonis, J. H., Gale, N. W., Van Aelst, L., Schlessinger, J., Wigler, M. H., and Bar-Sagi, D. (1993) *Science* **260**, 1338–1343
33. Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Sizeland, A. M., and Weinberg, R. A. (1993) *Nature* **363**, 45–51
34. de Vries-Smits, A. M. M., Burgering, B. M. T., Leevers, S. J., Marshall, C. J., and Bos, J. L. (1992) *Nature* **357**, 602–604
35. Robbins, D. J., Zhen, E., Cheng, M., Xu, S., Ebert, D., and Cobb, M. H. (1994) *Adv. Cancer Res.* **63**, 93–116
36. Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T., and Pelicci, P. G. (1992) *Cell* **70**, 93–104
37. Yokote, K., Mori, S., Hansen, K., McGlade, J., Pawson, T., Heldin, C.-H., and Claesson-Welsh, L. (1994) *J. Biol. Chem.* **269**, 15337–15343
38. Obermeier, A., Lammers, R., Weismuller, K.-H., Jung, G., Schlessinger, J., and Ullrich, A. (1993) *J. Biol. Chem.* **268**, 22963–22966
39. Pronk, G. J., McGlade, J., Pelicci, G., Pawson, T., and Bos, J. L. (1993) *J. Biol. Chem.* **268**, 5748–5753
40. Graus-Porta, D., Beerli, R. R., and Hynes, N. E. (1995) *Mol. Cell. Biol.* **15**, 1182–1191
41. Koland, J. G., O'Brien, K. M., and Cerione, R. A. (1990) *Biochem. Biophys. Res. Commun.* **166**, 90–100
42. Posada, J., and Cooper, J. A. (1992) *Science* **255**, 212–215
43. Horan, T., Wen, J., Arakawa, T., Liu, N., Brankow, D., Hu, S., Ratzkin, B., and Philo, J. S. (1995) *J. Biol. Chem.* **270**, 24604–24608
44. Culouscou, J.-M., Carlton, G. W., and Aruffo, A. (1995) *J. Biol. Chem.* **270**, 12857–12863
45. Dankort, D. L., Wang, Z., Blackmore, V., Moran, M. F., and Muller, W. J. (1997) *Mol. Cell. Biol.* **17**, 5410–25
46. Koland, J. G., and Cerione, R. A. (1988) *J. Biol. Chem.* **263**, 2230–2237
47. Mohammadi, M., Schlessinger, J., and Hubbard, S. R. (1996) *Cell* **86**, 577–587
48. Gamett, D. C., Pearson, G., Cerione, R. A., and Friedberg, I. (1997) *J. Biol. Chem.* **272**, 12052–12056
49. Marchionni, M. A., Goodearl, A. D. J., Chen, M. S., Bermingham-McDonogh, O., Kirk, C., Hendricks, M., Danehy, F., Misumi, D., Sudhalter, J., Kobayashi, K., Wroblewski, D., Lynch, C., Baldassare, M., Hiles, I., Davis, J. B., Hsuan, J. J., Totty, N. F., Otsu, M., McBurney, R. N., Waterfield, M. D., Stroobant, P., and Gwynne, D. (1993) *Nature* **362**, 312–318